# Differential expression of platelet activation markers, CD62P and CD63, after exposure to breast cancer cells treated with *Kigelia africana*, *Ximenia caffra* and *Mimusops zeyheri* seed oils *in vitro*

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**Abstract**

Cancer patients, including breast cancer patients, live in a hypercoagulable state. Chemo- and hormone- therapy used in the treatment of breast cancer increases the risk of thrombosis. Due to differences in health care services between developed and developing countries, the survival rate of women with breast cancer in developing countries is low. Consequently, ethnomedicines are used and their efficacy as potential alternatives are being scientifically explored. The seed oils of *Kigelia africana*, *Ximenia caffra* and *Mimusops zeyheri* have anti-proliferative effects on hormone-dependent (MCF-7) and cytotoxic effects on hormone-independent (MDA-MB-231) breast cancer cells. In this study, we determined if these seed oils reduce the thrombogenic ability of breast cancer cells by measuring the platelet surface expression of the activation-specific antigens CD62P and CD63. MDA-MB-231 and MCF-7 cells were pre-treated with the seed oils before being exposed to whole blood of human female volunteers. An increase in CD62P and CD63 expression following whole blood exposure to untreated breast cancer cells was observed. Treated MDA-MB-231 cells reduced CD62P and CD63 expression while treated MCF-7 cells increased CD62P and decreased CD63 expression. *Kigelia africana*, *Ximenia caffra* and *Mimusops zeyheri* seed oils are able to reduce the thrombogenic ability of MDA-MB-231 breast cancer cells.

**Key words:** breast cancer; thrombosis; seed oils; ethnomedicine

## Introduction

The relationship between cancer and thrombosis was established in the 1900s by Armand Trousseau when he observed malignancy in some patients after they had suffered thrombotic episodes (1, 2). Venous thromboembolism (VTE) is a common complication in cancer patients (2-4), with the likelihood of VTE occurrence being four- to seven- times higher in cancer patients than in non-cancer patients (5). This high prevalence of thrombi in oncology patients has been attributed to the activation of the patient's haemostatic system. Tumors can activate the host's haemostatic system through multiple mechanisms (5). One such mechanism entails the production of tissue factor (TF) that has been shown to play an important role in the initiation of the coagulation process (6). TF is a transmembrane glycoprotein that binds to factor VII/VIIa resulting in the activation of the extrinsic pathway of the clotting cascade to generate thrombin and eventually fibrin (6, 7). Reports have shown that cancer cells have the ability to release vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) (4, 7). These pro-angiogenic cytokines not only upregulate TF production but also enhance the expression of endothelial cell adhesion molecules that activate localized clotting factors, thereby promoting thrombosis formation (2).

Platelets mainly function to regulate hemostasis (8) and hence they are central to the formation of thrombi (9, 10). These anucleate blood cell fragments (2-4 μm) generated from megakaryocytes in the bone marrow, contain a variety of granules and proteins that equip them for their function (4, 8, 10, 11). For instance upon stimulation by TF, platelets release glycoproteins and coagulation factors from alpha granules as well as calcium ions, serotonin and ATP from dense granules to facilitate thrombus formation

(12). Interestingly TF-activated platelets also express P-selectin (CD-62P) and β-thromboglobulin, proteins shown to be important for tumor growth and metastasis (13, 14). CD62P (α-granule membrane protein) together with CD63, a lysosomal integral membrane protein, are often used for the detection of platelet activation in investigations using flow cytometry (15-17).

The most common cancer in women worldwide is breast cancer (~ 25%) with 2.09 million cases and 627 000 deaths reported in 2018 (18). Current treatment for breast cancer includes hormone therapy, chemotherapy (including neo adjuvant and adjuvant chemotherapy), radiotherapy and surgical removal of tumor tissue (19-21). Although chemotherapy in addition to surgery has proved useful in the treatment of breast cancer since it decreases the risk of recurrence and improves survival odds, it can cause severe side effects (22). Chemotherapy, which induces apoptosis in highly proliferative cells (23), also targets healthy cells and has subsequently been implicated in increasing the risk of thrombosis (24, 25). A thrombotic risk also exists for women on hormone replacement therapy (26). This risk is increased 1.5 to 7.1 fold when hormone therapy is used as adjuvant therapy in women with breast cancer following surgery (24, 27). It is therefore clear that current therapeutic strategies are not ideal and hence the search for better management approaches has to continue.

Besides present day cancer treatment being inadequate, it is also inaccessible due to unaffordable costs to many cancer patients. In developing countries, approximately 75% of women diagnosed with breast cancer are in clinical stages III and IV, whereas in North America 70% of newly diagnosed women with breast cancer are in clinical stages 0 and 1 (28). Five-year survival comparative rates estimate 12% from different

parts of Africa to almost 90% in North America (29). Reasons for these huge discrepancies include cultural barriers, and the lack of infrastructure and resources for early detection, proper diagnosis and treatment (18, 28, 29). As a result women with breast cancer in low- and middle-income countries are frequently misdiagnosed and/or receive insufficient treatment (28). In these communities ethnomedicine has been explored as a potential alternative to help women with breast cancer (22).

Ethnomedicine has historically been used for the prevention and treatment of various diseases (30). For instance Hodgkin's lymphoma and leukemia are treated with compounds isolated from the Madagascar periwinkle plants (*Vinca rosea*) – Vinblastine and Vincristine (31, 32), while Paclitaxel, originally extracted from the bark of the Pacific Yew, *Taxus brevifolia* Nutt, is frequently used in the treatment of ovarian and breast cancer (32, 33). However the use of some of these herbiceuticals has been controversial. A concern has been raised over a possible Paclitaxel-induced pro-thrombotic risk as a number of reports of thrombotic episodes in cancer patients being treated with this chemotherapy plant alkaloid have been reported (34, 35).

*Kigelia africana* (*K. africana*), *Ximenia caffra* (*X. caffra*) and *Mimusops zeyheri* (*M. zeyheri*) are commonly found in southern Africa (36). Traditional healers use various parts of these trees for the treatment of a number of diseases (36). For example, a tea brewed from the powdered bark of *K. africana* has been shown to have analgesic and anti-inflammatory (37) and, more recently, anti-neoplastic (38, 39) effects. Similarly the bark of *M. zeyheri* have been used to treat ulcers and wounds, while its roots have been used to treat candidiasis (40). Proliferation of murine RAW 264.7 macrophage

cells are suppressed by extracts of the *X. caffra* leaf suggesting anti-inflammatory properties of this plant species (41).

The health benefits of plants are not limited to extracts of leaf or bark material, but also includes that of seeds (42). Seeds of plants such as *Glycine max* (soya bean), *Gossipium hirstum* (cotton seed), *Helianthus annuus* (sunflower) and *Sesamun indicum* (sesame seed) contain essential amino acids, fatty acids and vitamins (42) that may afford some of the medicinal properties of seeds. Subsequently oils extracted from seeds have been shown to possess anti-inflammatory (43) and anti-proliferative (44-47) properties. Anti-cancer effects of some seed oils have been indicated by (i) a decrease in the production of vascular endothelial growth factor (VEGF) by breast cancer cells treated with Pomegranate (*Punica granatum* L.) seed oil (43), (ii) inhibition of melanoma cell growth by *Pterodon pubescens* Benth. seed oil (44), (iii) the prevention of tumor cell progression through the cell cycle by *Litsea cubeba* and *Portulaca oleracea* seed oils (45-47), and (iv) the decrease of proliferation of human colon adenocarcinoma (Caco-2) cells by *K. africana*, *X. caffra* and *M. zeyheri* seed oils (36).

While the literature suggests seed oils of some plants to have prominent anti-tumor potential, data from our laboratory indicated that these results need to be interpreted with caution. We recently showed that *K. africana*, *M. zeyheri* and *X. caffra* seed oils, while having anti-proliferative effects on hormone-dependent breast cancer cells (MCF-7), exert cytotoxic effects on hormone-independent breast cancer cells (MDA-MB-231) (48). Given the high dependency on ethnomedicines to manage diseases in low- to middle-income countries, it is evident that more research is required to affirm

the medicinal benefit of seed oils. In the present study an investigation was conducted to determine if *K. africana*, *X. caffra* and *M. zeyheri* seed oils could reduce the thrombogenic ability of breast cancer cells by measuring the ability of these seed oils to inhibit breast cancer cell-induced activation of platelets. Two human breast cancer cell lines (MDA-MB-231 and MCF-7) were pre-treated with the respective seed oils prior to being exposed to whole blood (WB) of healthy human female volunteers. The anti-thrombogenic capacity of the seed oils were determined by measuring the platelet surface expression of the activation-specific antigens CD62P and CD63.

## Materials and Methods

### *Seed source*

*K. africana* seeds were obtained from the ripe fruits of trees in Gutu District, Chitsa Communal area, Zimbabwe. *M. zeyheri* seeds were harvested from fruits of trees found in the Gokwe District, in North West Zimbabwe and in the Machakata Community Forest area of the Mapfungautsi Plateau, while *X. caffra* seeds were collected from ripe fruit of the Zhombe District, Zimbabwe.

### *Seed oil extraction and storage*

Seeds were deshelled and crushed in a blender (Waring; Lasec Pty Ltd, Johannesburg, South Africa). Hexane was used to extract the oil from each of the seed meals as described by Yamasaki *et al.* (49). In short, 800 mL of hexane was added to 160 g of each seed meal and then mixed overnight using a magnetic stirrer (VELP Scientifica, Lombardy – Italy) at room temperature. The mixture was then passed through filter paper (Whatman No.1) and the hexane evaporated off using a

rotary vacuum at 65$^{o}$C. The extracted oils were kept in brown sample bottles at -20 $^{o}$C before use (49).

*Cell culture*

A Human Ethics Waiver Certificate (W-CJ-150422-1) was obtained from the Human Research Ethics Committee, University of the Witwatersrand, Johannesburg. The two human breast cancer cell lines (MDA-MB-231 and MCF-7) used in the experiments were sourced from ATCC (Virginia, USA) and donated by Dr R Duarte (School of Clinical Medicine, University of the Witwatersrand). MCF-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 5% fetal bovine serum (FBS) and 100 μg/mL streptomycin, 100 units/mL penicillin. MDA-MB-231 cells were cultured in DMEM/Ham's F-12 nutrient mixture with 10% FBS and 100 μg/mL streptomycin, 100 units/mL penicillin. Both cell lines were incubated at 37$^{o}$C in a humidified atmosphere of 5% $CO_2$.

*Seed oil concentrations and treatment of cells*

Stock solutions (10 mg/mL) of the seed oils were prepared in 100% absolute alcohol and diluted in culture medium to reach the desired concentration (120 μg/mL). The concentration of absolute alcohol in the culture medium was not more than 5%. The control consisted of 100% absolute alcohol diluted in culture medium to obtain a 5% final solution.

Confluent cells were harvested in 0.25% trypsin/EDTA. MCF-7 cells (P44) and MDA-MB-231 cells (P19) were plated in 24 well plates at 6.3 x $10^4$ cells/well and 3.1 x $10^4$ cells/well, respectively in their specific media. Both cell lines were incubated at 37$^{o}$C

in a humidified atmosphere of 5% $CO_2$ for 24 h. The cell culture media from each well was then removed and cells rinsed with Dulbecco's phosphate-buffered saline (DPBS). Cells were treated with 200 µL of the seed oil at a concentration of 120 µg/mL in matched culture media and incubated at 37$^{o}$C in a humidified atmosphere of 5% $CO_2$ for 48 h. In all experiments untreated and diluent treated cells were included as controls.

*Blood collection*

Ethical clearance was obtained from the University of the Witwatersrand's Human Ethics Committee (Clearance Certificate Number M160211) for the collection of blood from healthy female participants between the ages of 19-30 years (n = 8) (23.6±2.8 years, mean age ± SD). Exclusion criteria for participants included pregnancy, contraceptive use, smoking, previous history of cancer, presence of autoimmune diseases, immunodeficiency and the use of anti-platelet/anti-coagulation medication. After giving informed consent, blood was collected from participants on days 5.4±2.7 (mean days ± SD) of their menstrual cycle. Circulating estrogen and progesterone levels are low between days 1 and 10 of the menstrual cycle, consequently the potential effects of these hormones on breast cancer cells are limited (50).

Peripheral blood was obtained by venipuncture into 0.109M (3.2%) sodium citrate vacutainers (Lasec, South Africa). The first 2 mL of blood drawn was discarded to minimize the inclusion of mechanically activated platelets in the study (51). Blood samples were processed within 1 h of collection.

*Blood co-culture and platelet labeling*

After the 48 h incubation period, media were removed from the wells. The remaining cells were then incubated with 200 µL WB for 2.5 min. For analysis of platelets, 100 µL WB was removed from the wells and incubated in 1 mL cold ammonium chloride ($NH_4Cl$) erythrocyte lysis buffer for 10 min at room temperature. Samples were subsequently centrifuged at 200 × g for 5 min and the supernatant discarded. The pellet was gently resuspended in 150 µL cold Tyrode's buffer and incubated with their respective pre-titrated antibodies for 15 min: 10 µL allophycocyanin (APC)-conjugated mouse anti-human CD41a (559777; BD Pharmingen) was used as a tagging antibody for platelets whilst 10 µL fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD62P (555523; BD Pharmingen) and 2.5 µL phycoerythrin cyanine (PE-Cy) mouse anti-human CD63 (561982; BD Pharmingen) were used to detect platelet activation. The samples were then fixed with 1% paraformaldehyde (PFA) for 10 min and subsequently centrifuged at 200 × g for 5 min and the supernatant discarded. The pellet was again gently resuspended in 500 µL cold Tyrode's buffer and transferred into tubes appropriate for use in flow cytometry. Platelets activated by 0.1U thrombin were used as a positive control (50, 52).

*Flow cytometry*

Samples were incubated on ice before data acquisition on an LSRFortessa (BD Biosciences) equipped with four lasers (Blue, Red, Violet and UV). Events (100 000 events per sample) were acquired and pre-analyzed using the BD Biosciences FACSDiva software. The events were recorded at a forward scatter voltage of 240V and a side scatter voltage of 210V. Compensation controls included unlabeled WB samples and pre-titrated single antibody staining with CD41a-APC, CD62P-FITC and CD63 PE-Cy to determine the levels of auto fluorescence and fluorescence overlap.

All samples were analyzed in duplicate. Exported Flow cytometry Standard (FCS) files were analyzed using FlowJo V10 (FlowJo, LLC, Ashland, OR 97520, USA).

Results are expressed as percentage change in CD62P and CD63 expression after exposure to non-treated/treated breast cancer cells relative to WB alone and was calculated as follows:

$$\%\ change = \frac{Frequency\ of\ CD62P\ or\ CD63\ positive\ platelets\ from\ WB\ or\ WB\ exposed\ to\ untreated\ or\ treated\ breast\ cancer\ cells}{Frequency\ of\ CD62P\ or\ CD63\ positive\ platelets\ from\ WB\ alone} X\ 100$$

*Statistical data analysis*

The individual data of each participant is presented together with the average for the respective groups which is depicted as mean ± SD. Data were analyzed using SPSS (IBM SPSS Statistics for Windows, Version 25.0, Chicago, USA). All the numerical values were tested for normality using the Shapiro-Wilk Test. Differences between groups were evaluated using the two-way analysis of variance (ANOVA). A *p* value of <0.05 was considered significant.

## Results

*Flow Cytometry*

The gating strategy to detect platelet activation using representative data of unstained and stained untreated WB populations is shown in Figure 1.

Using representative data, scatter plots showing the expression of platelet activation markers after WB was exposed to untreated and treated breast cancer cells (MDA-MB-231), are shown in Figure 2.

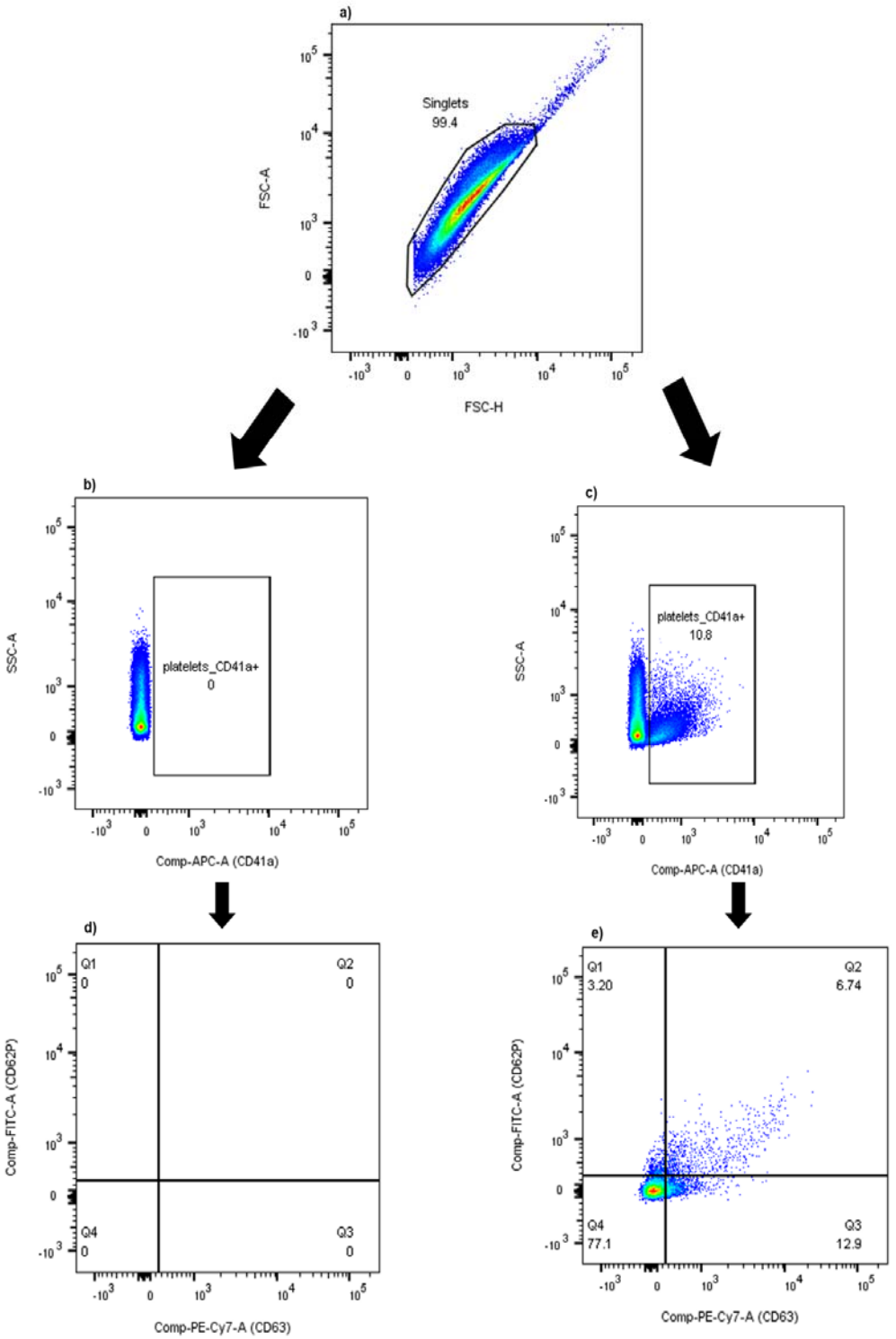


**Figure 1**. Gating strategy used to detect platelet activation using unstained (a, b, d) and stained (c, e) WB. (a) A singlet population of WB was selected using forward scatter height vs. forward scatter area density plot. To discriminate platelets from other cells and debris, a single platelet gate was positioned on the basis of side scatter and platelet marker (APC-conjugated mouse anti-human CD41a) (b & c). Platelet activation was then determined using FITC-conjugated mouse anti-human CD62P and phycoerythrin cyanine (PE-Cy) mouse anti-human CD63 antibodies as shown in the quadrant plot in (d) and (e).

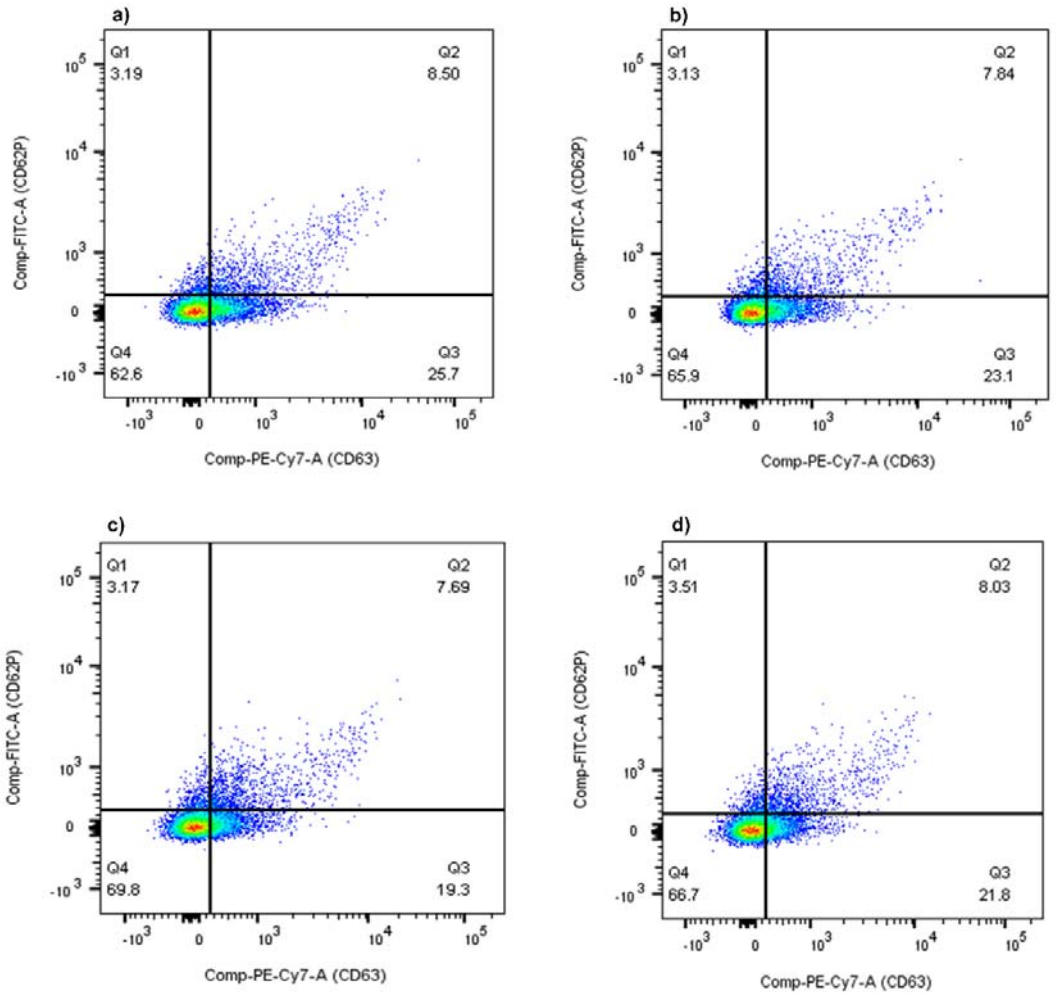


**Figure 2**. Scatter plots showing the expression of platelet activation markers after WB was exposed to untreated and treated MDA-MB-231 breast cancer cells. (a) WB exposed to untreated cells, (b) WB exposed to cells treated with KA seed oil, (c) WB exposed to cells treated with XC seed oil and (d) WB exposed to cells treated with MZ seed oil.

*Percentage change of CD62P expression after WB was exposed to untreated and treated MDA-MB-231 breast cancer cells*

Out of eight participants, platelets of five participants (P1, P3, P4, P6 and P8) showed an increase in the percentage change of CD62P expression after their WB was exposed to untreated MDA-MB-231 breast cancer cells (Figure 3a), while that of one participant (P5) was reduced and two others (P2 and P7) basically remained unchanged (Figure 3a). The increase in the percentage change of CD62P expression in the five participants was not observed after their WB was exposed to KA-treated MDA-MB-231 breast cancer cells (Figure 3b), while the percentage change of CD62P expression was increased in P5 and continued to be unchanged in P2 and P7. As a group, the average percentage change of CD62P expression increased by 24.45% when the WB was exposed to untreated MDA-MB-231 breast cancer cells, but was reduced when WB was exposed to KA-treated MDA-MB-231 breast cancer cells. However these changes did not reach statistical significance.

Similarly, the percentage change of CD62P expression levels that were previously high in the five participants following exposure of their WB to untreated MDA-MB-231 breast cancer cells (Figure 3a), were decreased when their WB was exposed to XC-treated MDA-MB-231 breast cancer cells (Figure 3c). Interestingly P5 that previously showed a reduced percentage change of CD62P expression when exposed to untreated MDA-MB-231 breast cancer cells, displayed a further reduction when exposed to XC-treated MDA-MB-231 breast cancer cells (Figure 3c), while P2 and P7 that exhibited an unchanged percentage change of CD62P expression when exposed to untreated MDA-MB-231 breast cancer cells, showed a differential response to XC-treated MDA-MB-231 breast cancer cells with P2 showing an increase and P7 a

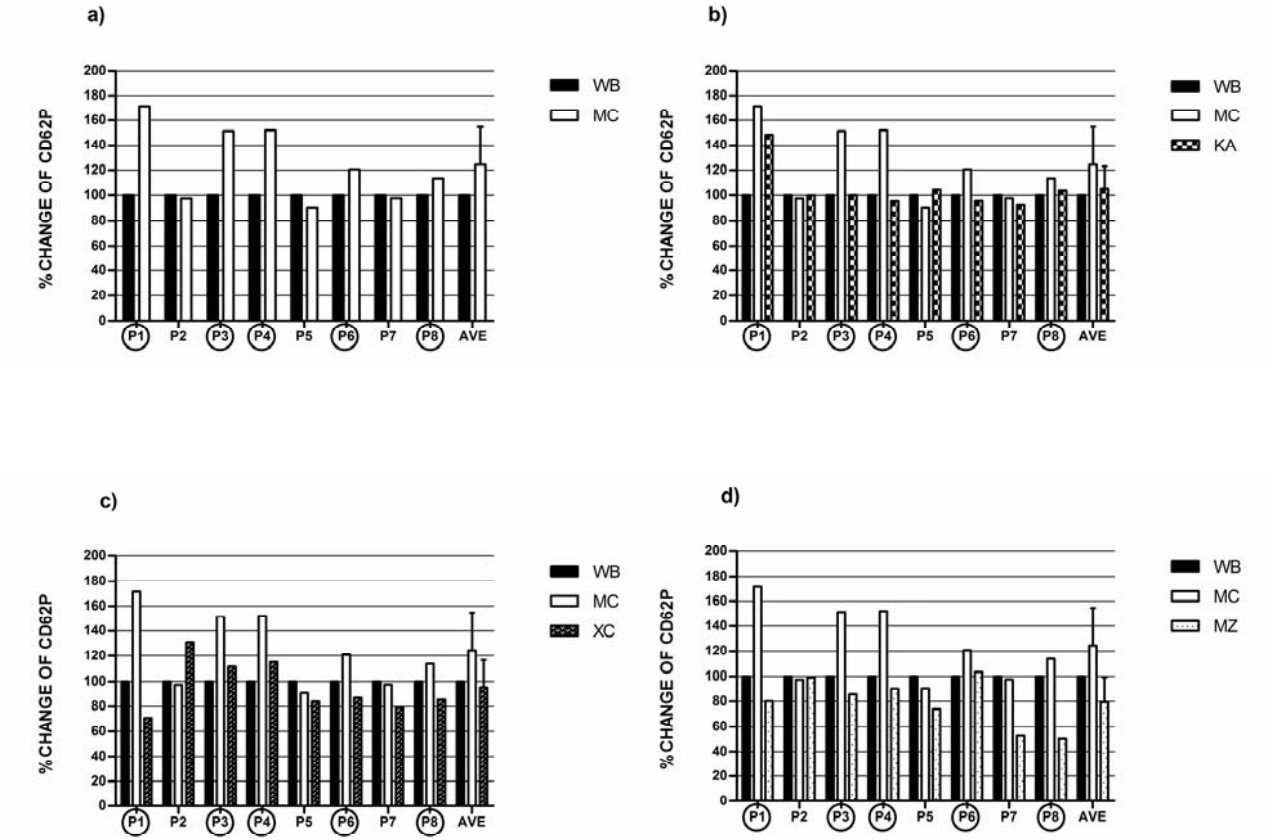


**Figure 3**. Graphs showing percentage change of the platelet activation marker, CD62P, of each participant, as well as the group average, after their WB was exposed to untreated (a) and treated MDA-MB-231 breast cancer cells (b, c, d). WB = WB only, MC = WB exposed to untreated breast cancer cells, KA = WB exposed to breast cancer cells treated with KA seed oil, XC = WB exposed to breast cancer cells treated with XC seed oil and MZ = WB exposed to breast cancer cells treated with MZ seed oil. P1, P2, P3 … = Participant 1, Participant 2, Participant 3, etc. Encircled participants indicate an increase in CD62P expression after their WB was exposed to untreated MDA-MB-231 breast cancer cells.

decrease in the percentage change of CD62P expression (Figure 3c). On average, WB exposure to untreated MDA-MB-231 breast cancer cells led to an increase in the percentage change of CD62P expression levels that were not seen when the WB was exposed to XC-treated MDA-MB-231 breast cancer cells (Figure 3c).

Exposure of the WB of the participants to MZ-treated MDA-MB-231 breast cancer cells, yielded comparable results with P1, P3, P4, P6 and P8 showing reduced, P2 unchanged, and P5 and P7 a further reduction in the percentage change of CD62P expression (Figure 3d). On average, the percentage change of CD62P expression of the group decreased from 124.45% (the percentage change of CD62P expression from WB exposed to untreated MDA-MB-231 breast cancer cells) to 79.47% after WB exposure to MZ-treated MDA-MB-231 breast cancer cells (Figure 3d).

The box plot shown in Figure 4 shows the pooled data of all the participants' response to the platelet activation marker CD62P to untreated and treated MDA-MB-231 breast cancer cells. Overall, MZ-treated MDA-MB-231 breast cancer cells had a decrease in percentage change of CD62P expression.

*Percentage change of CD62P expression after WB was exposed to untreated and treated MCF-7 breast cancer cells*

Out of the same eight participants, the percentage change of CD62P expression of four participants increased after WB exposure to untreated MCF-7 breast cancer cells relative to WB alone (P3, P4, P5 and P6), while that of two participants (P1 and P2) were unchanged and another two (P7 and P8) were decreased (Figure 5a). The increase in the percentage change of CD62P expression observed in participants P3,

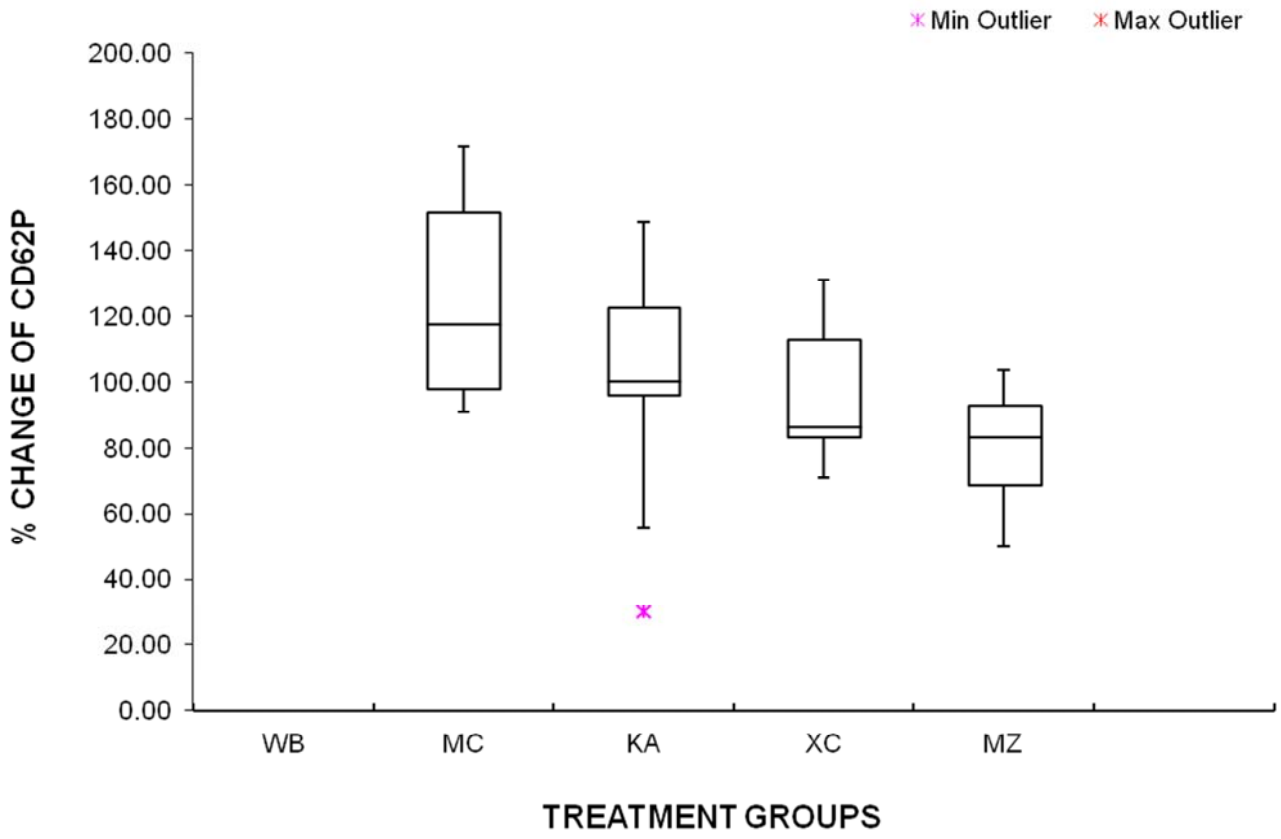


**Figure 4.** Box plot showing the pooled data of all the participants' response to the platelet activation marker CD62P to untreated and treated MDA-MB-231 breast cancer cells. WB = WB only, MC = WB exposed to untreated breast cancer cells, KA = WB exposed to breast cancer cells treated with KA seed oil, XC = WB exposed to breast cancer cells treated with XC seed oil and MZ = WB exposed to breast cancer cells treated with MZ seed oil.

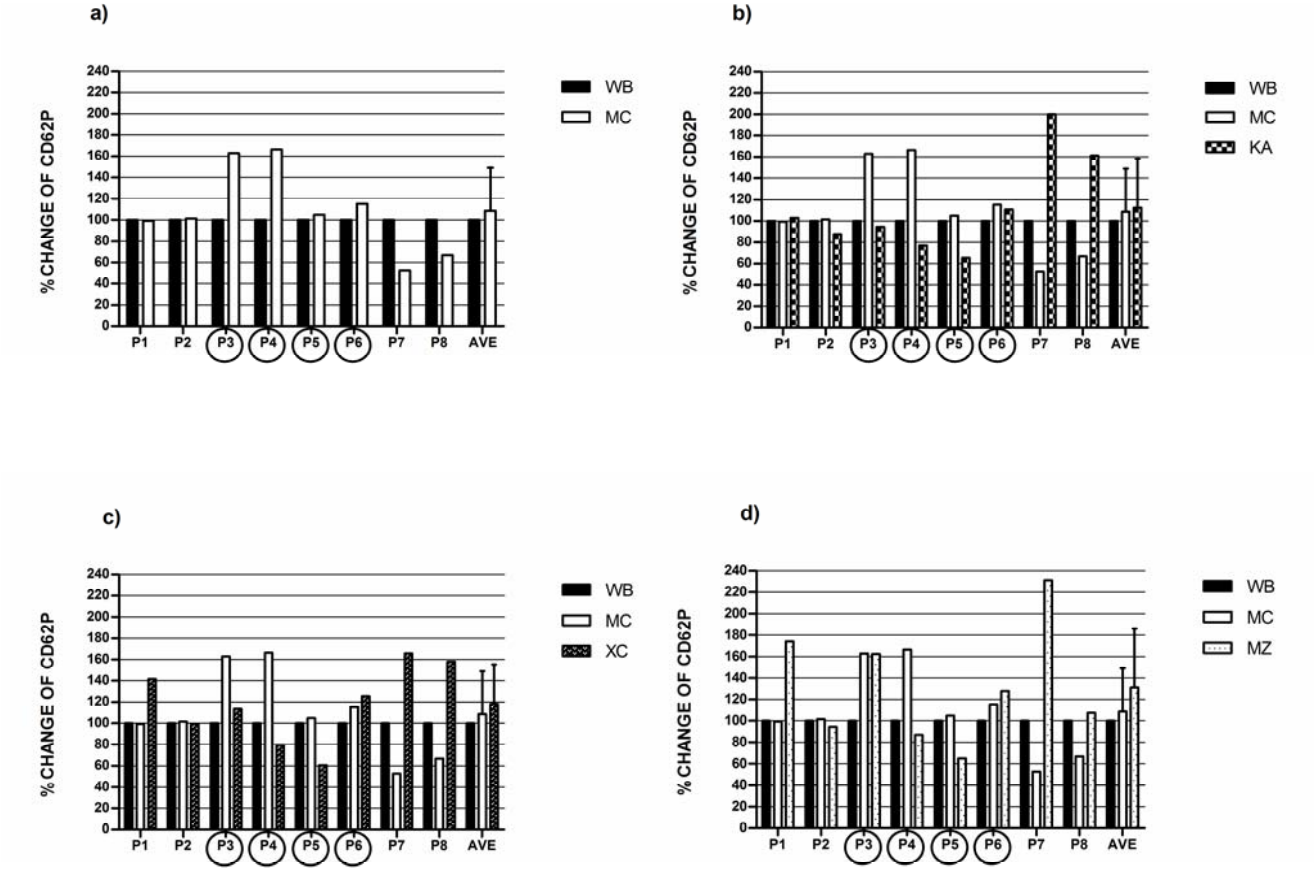


**Figure 5.** Graphs showing percentage change of the platelet activation marker, CD62P, of each participant, as well as the group average, after their WB was exposed to untreated (a) and treated MCF-7 breast cancer cells (b, c, d). WB = WB only, MC = WB exposed to untreated breast cancer cells, KA = WB exposed to breast cancer cells treated with KA seed oil, XC = WB exposed to breast cancer cells treated with XC seed oil and MZ = WB exposed to breast cancer cells treated with MZ seed oil. P1, P2, P3 … = Participant 1, Participant 2, Participant 3, etc. Encircled participants indicate an increase in CD62P expression after their WB was exposed to untreated MCF-7 breast cancer cells.

P4, P5 and P6, was not evident after WB was exposed to KA-treated MCF-7 breast cancer cells (Figure 5b). The percentage change of CD62P expression was unchanged in P1 and decreased in P2 when their WB was exposed to KA-treated MCF-7 breast cancer cells. In contrast, the percentage change of CD62P expression in P7 and P8 was dramatically stimulated when their WB was exposed to KA-treated MCF-7 breast cancer cells. As a group, the average percentage change of CD62P expression was increased when the WB was exposed to untreated and KA-treated MCF-7 breast cancer cells. This increase was found not to be statistically significant.

The percentage change of CD62P expression levels that were previously high in the four participants following exposure of their WB to untreated MCF-7 breast cancer cells (Figure 5a), were decreased (with the exception of P6) when their WB was exposed to XC-treated MCF-7 breast cancer cells (Figure 5c). There was an increase in the percentage change of CD62P expression for P1, P6, P7 and P8 while the percentage change of CD62P expression remained unchanged for P2 when their WB was exposed to XC-treated MCF-7 breast cancer cells. As a group, the average percentage change of CD62P expression increased by 9.35% after WB exposure to XC-treated MCF-7 breast cancer cells. This increase was found not to be statistically significant.

The percentage change of CD62P expression levels that were previously high in the four participants following exposure of their WB to untreated MCF-7 breast cancer cells (Figure 5a), were decreased (with the exception of P6) when their WB was exposed to MZ-treated MCF-7 breast cancer cells (Figure 5d). There was an increase in the percentage change of CD62P expression for P1, P6, P7 and P8 while the

percentage change of CD62P expression remained unchanged for P2 when their WB was exposed to MZ-treated MCF-7 breast cancer cells. As a group, the average percentage change of CD62P expression increased by 22.45% after WB exposure to MZ-treated MCF-7 breast cancer cells.

The box plot shown in Figure 6 shows the pooled data of all the participants' response to the platelet activation marker CD62P to untreated and treated MCF-7 breast cancer cells. Overall, MZ-treated MCF-7 breast cancer cells had an increase in percentage change of CD62P expression.

*Percentage change of CD63 expression after WB was exposed to untreated and treated MDA-MB-231 breast cancer cells*

Investigating the percentage change of CD63 expression in the same eight participants showed that four participants had an increase in the percentage change of CD63 expression levels after WB exposure to untreated MDA-MB-231 breast cancer cells relative to WB alone (P5, P6, P7 and P8), while that of one participant (P2) was unchanged and another three (P1, P3 and P4) were decreased (Figure 7a). The increase in the percentage change of CD63 expression in the four participants was not observed after their WB was exposed to KA-treated MDA-MB-231 breast cancer cells (Figure 7b) while the percentage change of CD63 expression was increased in P2 and P3 but decreased in P1 and P4. As a group, the average percentage change of CD63 expression increased when the WB was exposed to untreated MDA-MB-231 breast cancer cells but was reduced when the WB was exposed to KA-treated MDA-MB-231 breast cancer cells.

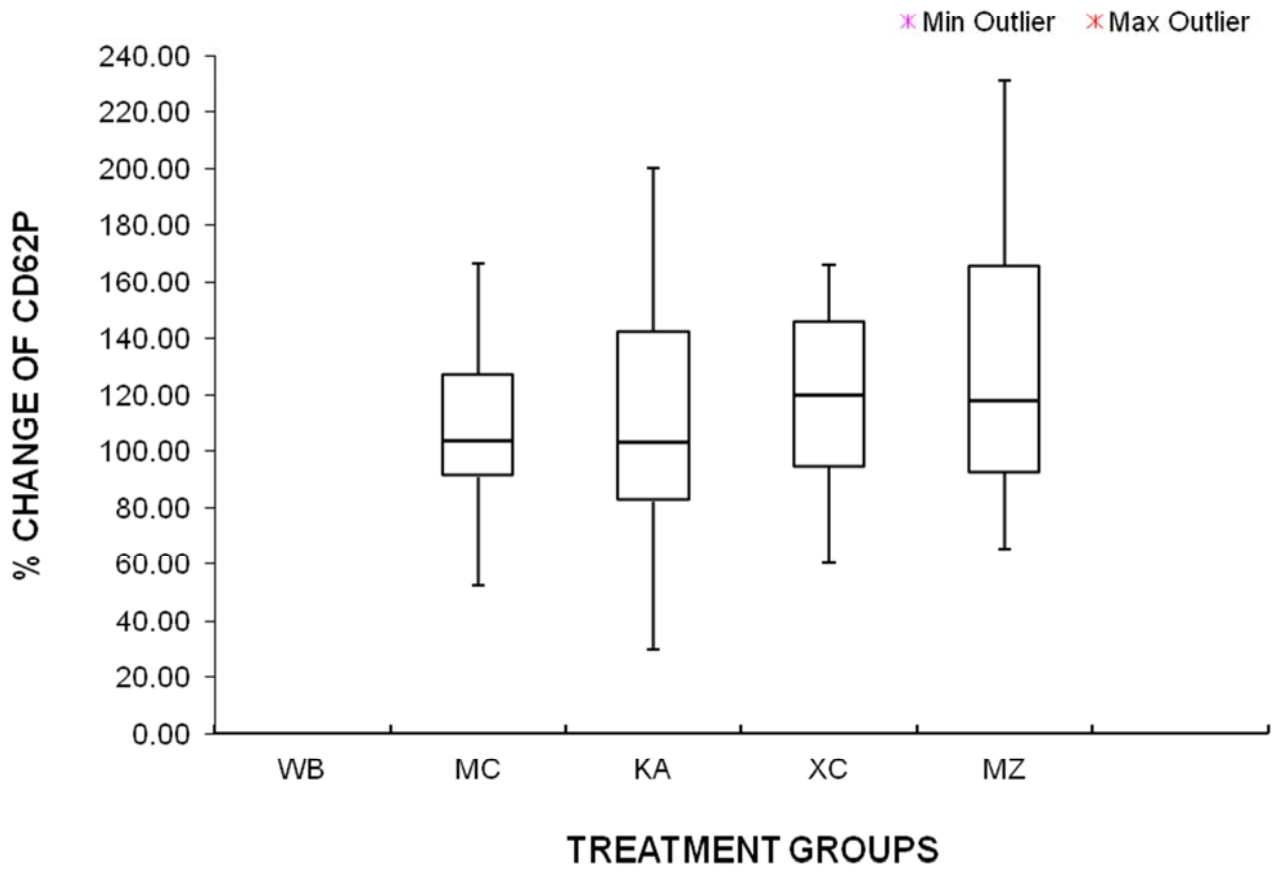


**Figure 6**. Box plot showing the pooled data of all the participants' response to the platelet activation marker CD62P to untreated and treated MCF-7 breast cancer cells. WB = WB only, MC = WB exposed to untreated breast cancer cells, KA = WB exposed to breast cancer cells treated with KA seed oil, XC = WB exposed to breast cancer cells treated with XC seed oil and MZ = WB exposed to breast cancer cells treated with MZ seed oil.

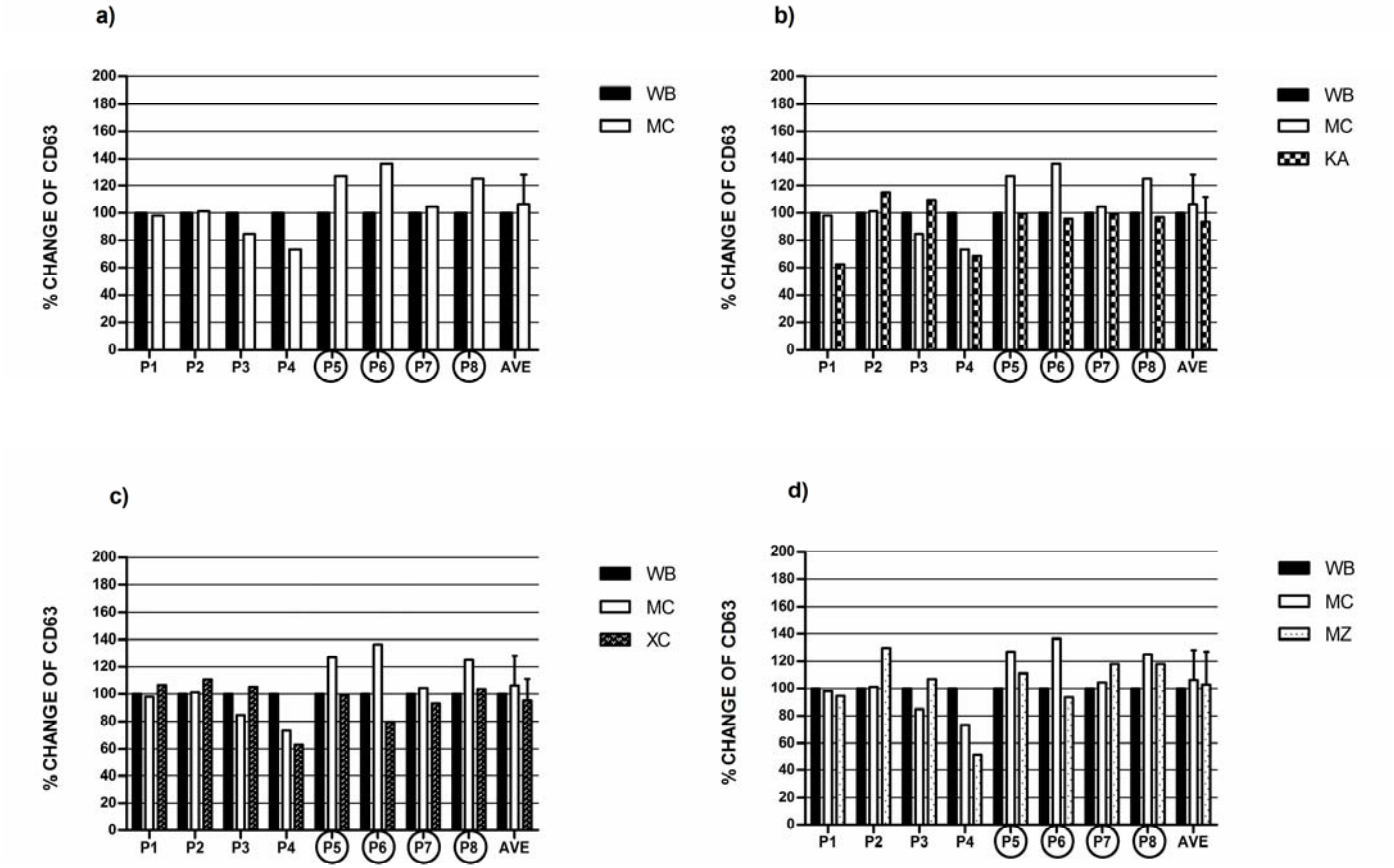


**Figure 7.** Graphs showing percentage change of the platelet activation marker, CD63, of each participant, as well as the group average, after their WB was exposed to untreated (a) and treated MDA-MB-231 breast cancer cells (b, c, d). WB = WB only, MC = WB exposed to untreated breast cancer cells, KA = WB exposed to breast cancer cells treated with KA seed oil, XC = WB exposed to breast cancer cells treated with XC seed oil and MZ = WB exposed to breast cancer cells treated with MZ seed oil. P1, P2, P3 … = Participant 1, Participant 2, Participant 3, etc. Encircled participants indicate an increase in CD63 expression after their WB was exposed to untreated MDA-MB-231 breast cancer cells.

Similarly, the percentage change of CD63 expression levels that were previously high in the four participants following exposure of their WB to untreated MDA-MB-231 breast cancer cells (Figure 7a), were decreased when their WB was exposed to XC-treated MDA-MB-231 breast cancer cells, while the percentage change of CD63 expression was increased for P1, P2 and P3 but decreased for P4 (Figure 7c). On average, WB exposure to untreated MDA-MB-231 breast cancer cells led to an increase in the percentage change of CD63 expression levels that were not seen when the WB was exposed to XC-treated MDA-MB-231 breast cancer cells (Figure 7c).

The percentage change of CD63 expression levels that were previously high in the four participants following exposure of their WB to untreated MDA-MB-231 breast cancer cells (Figure 7a), were decreased (with the exception of P7) when their WB was exposed to MZ-treated MDA-MB-231 breast cancer cells (Figure 7d). There was an increase in the percentage change of CD63 expression for P2, P3 and P7, while the percentage change of CD63 expression was decreased for P1 when their WB was exposed to MZ-treated MDA-MB-231 breast cancer cells. As a group, the average percentage change of CD63 expression decreased after WB exposure to MZ-treated MDA-MB-231 breast cancer cells.

The box plot shown in Figure 8 shows the pooled data of all the participants' response to the platelet activation marker CD63 to untreated and treated MDA-MB-231 breast cancer cells. Overall, KA-treated MDA-MB-231 breast cancer cells had a decrease in percentage change of CD63 expression.

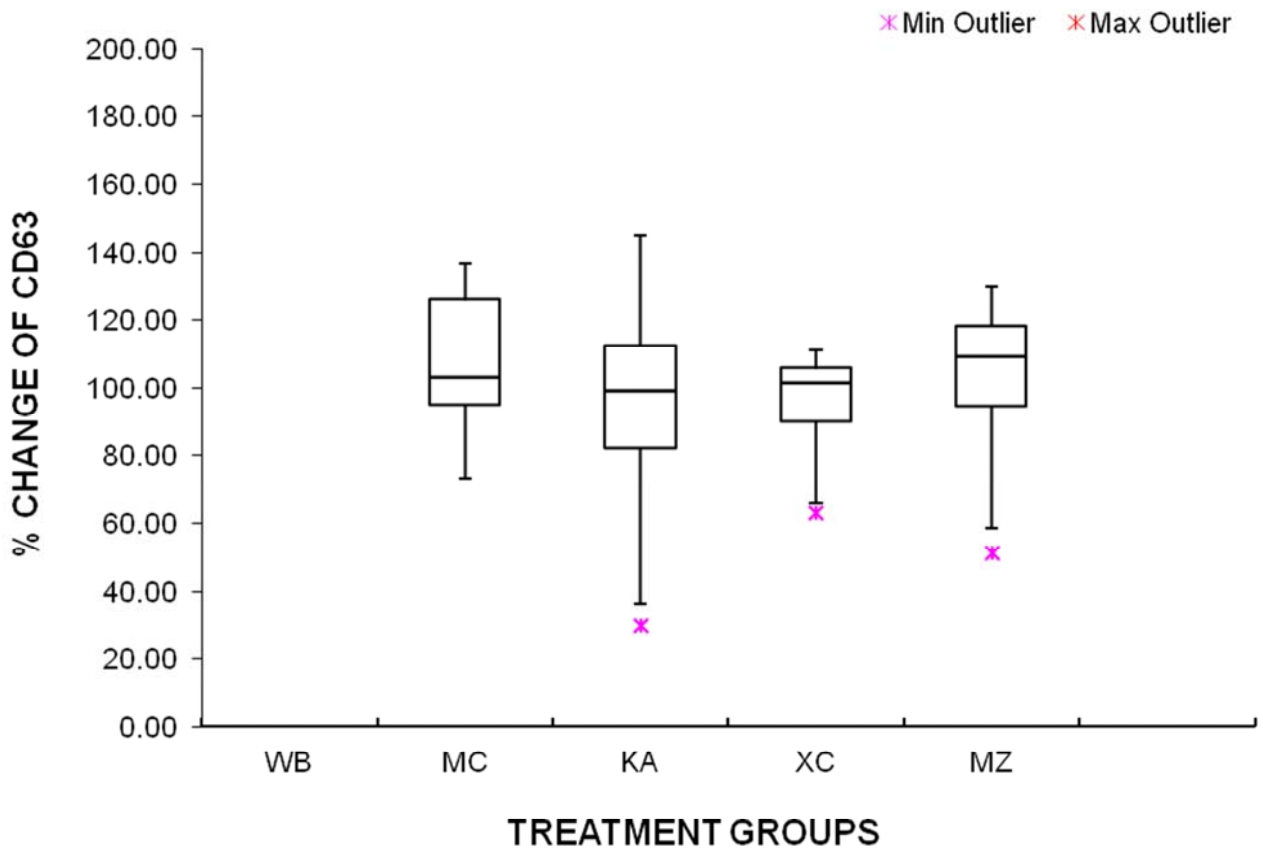


**Figure 8.** Box plot showing the pooled data of all the participants' response to the platelet activation marker CD63 to untreated and treated MDA-MB-231 breast cancer cells. WB = WB only, MC = WB exposed to untreated breast cancer cells, KA = WB exposed to breast cancer cells treated with KA seed oil, XC = WB exposed to breast cancer cells treated with XC seed oil and MZ = WB exposed to breast cancer cells treated with MZ seed oil.

*Percentage change of CD63 expression after WB was exposed to untreated and treated MCF-7 breast cancer cells*

Out of the same eight participants, platelets of five participants (P1, P2, P6, P7 and P8) showed an increase in the percentage change of CD63 expression after their WB was exposed to untreated MCF-7 breast cancer cells (Figure 9a) while the percentage change of CD63 expression remained unchanged in one participant (P3) and decreased in two other participants (P4 and P5). The increase in the percentage change of CD63 expression in the five participants was not observed after their WB was exposed to KA-treated MCF-7 breast cancer cells (Figure 9b) while the percentage change of CD63 expression was increased in P4, the percentage change of CD63 expression remained unchanged for P5 and decreased for P3. As a group, the average percentage change of CD63 expression increased when WB was exposed to untreated MCF-7 breast cancer cells but was reduced when WB was exposed KA-treated MCF-7 breast cancer cells.

Similarly, a decrease in the percentage change of CD63 expression was observed for these participants (P1, P2, P6, P7 and P8) including P3, while the percentage change of CD63 expression was increased in P4 and P5 after their WB was exposed to XC-treated MCF-7 breast cancer cells (Figure 9c). On average, the percentage change of CD63 expression decreased after WB exposure to XC-treated MCF-7 breast cancer cells (Figure 9c).

Exposure of the WB of the participants to MZ-treated MCF-7 breast cancer cells, yielded comparable results with P1, P2, P6, P7 and P8 showing a reduced percentage change of CD63 expression including P3, while P4 and P5 showed an increase in the

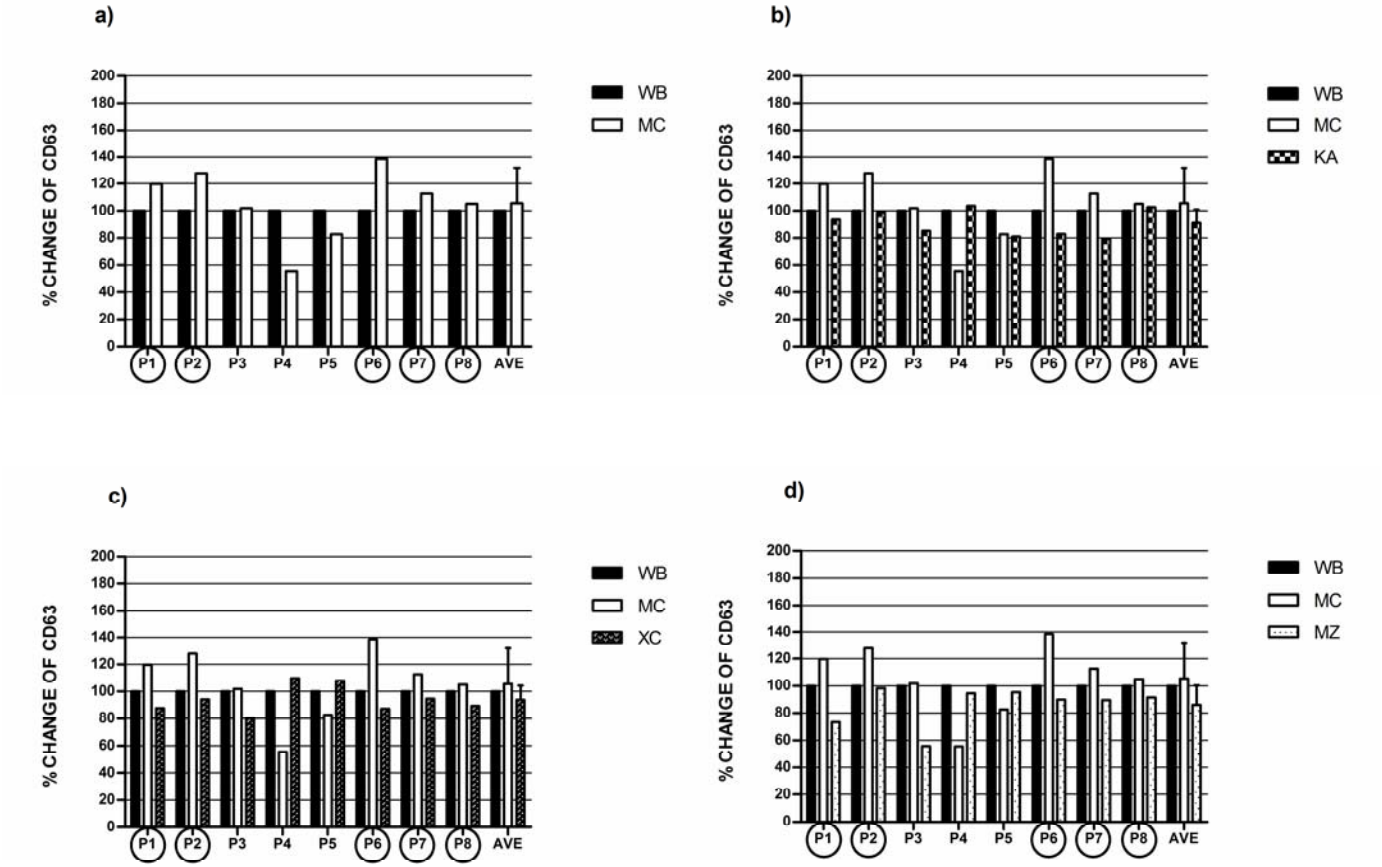


**Figure 9.** Graphs showing percentage change of the platelet activation marker, CD63, of each participant, as well as the group average, after their WB was exposed to untreated (a) and treated MCF-7 breast cancer cells (b, c, d). WB = WB only, MC = WB exposed to untreated breast cancer cells, KA = WB exposed to breast cancer cells treated with KA seed oil, XC = WB exposed to breast cancer cells treated with XC seed oil and MZ = WB exposed to breast cancer cells treated with MZ seed oil. P1, P2, P3 … = Participant 1, Participant 2, Participant 3, etc. Encircled participants indicate an increase in CD63 expression after their WB was exposed to untreated MCF-7 breast cancer cells.

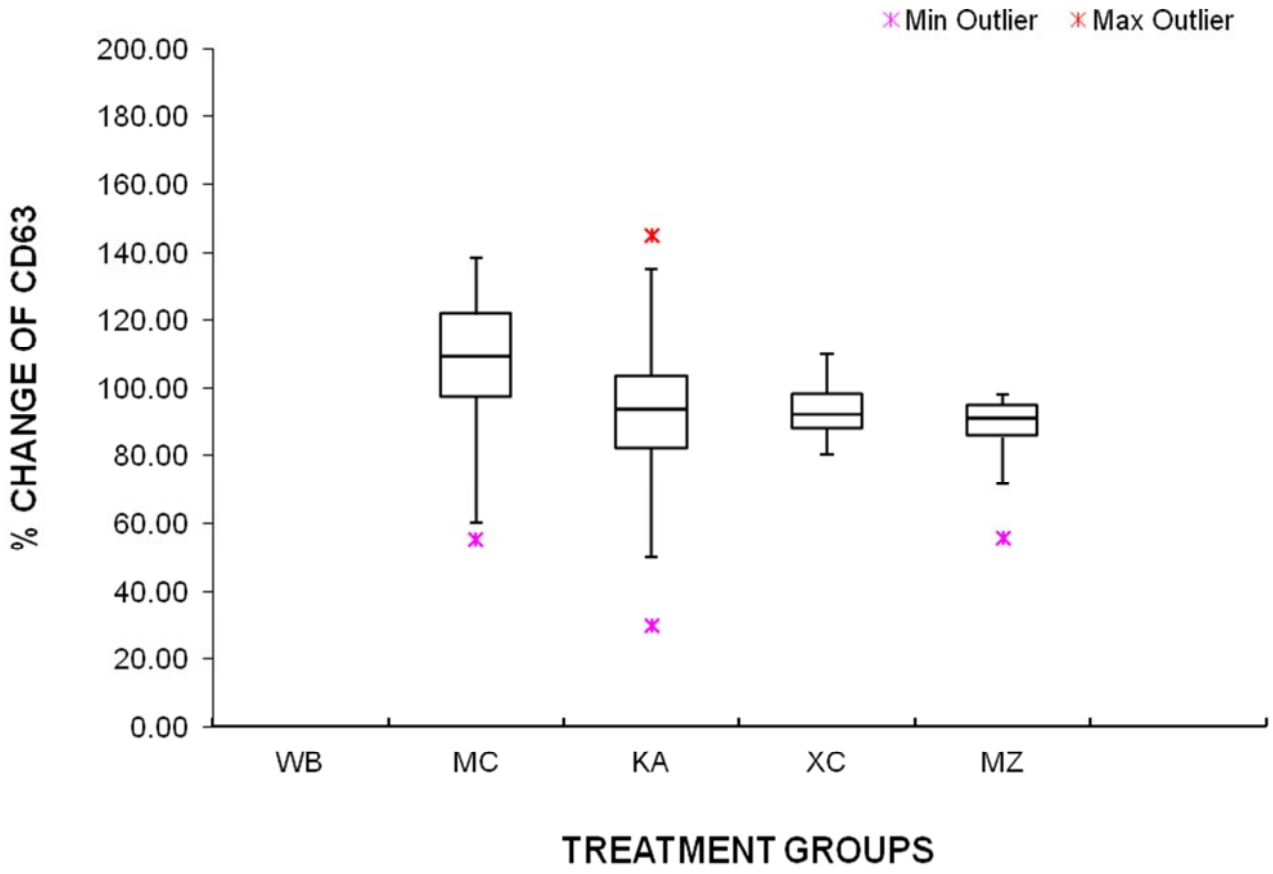


**Figure 10.** Box plot showing the pooled data of all the participants' response to the platelet activation marker CD63 to untreated and treated MCF-7 breast cancer cells. WB = WB only, MC = WB exposed to untreated breast cancer cells, KA = WB exposed to breast cancer cells treated with KA seed oil, XC = WB exposed to breast cancer cells treated with XC seed oil and MZ = WB exposed to breast cancer cells treated with MZ seed oil.

percentage change of CD63 expression (Figure 9d). On average, the percentage change of CD63 expression of the group decreased after WB exposure to MZ-treated MCF-7 breast cancer cells (Figure 9d).

The box plot shown in Figure 10 shows the pooled data of all the participants' response to the platelet activation marker CD63 to untreated and treated MCF-7 breast cancer cells. Overall, MZ-treated MCF-7 breast cancer cells had a decrease in percentage change of CD63 expression.

**Discussion**

Cancer patients, including breast cancer patients, live in a hypercoagulable state, either because cancer cells produce and secrete TF, cancer procoagulant and fibrinolytic proteins (25, 53, 54) or due to the treatments cancer patients receive (24, 27, 55). Platelets are activated by cancer cells either by the above-mentioned secreted factors or by the adhesion of the cancer cells to circulating platelets (4, 8, 56). Once activated, important granule membrane molecules, such as P-selectin (CD62P) and CD63 become evident on the platelet cell surface which subsequently aid tumor growth and metastasis (13, 14, 57). Consequently, platelets play an important role in cancer associated thrombosis and cancer spread (13, 58).

In this preliminary investigation, we found differential expression of the platelet activation markers, CD62P and CD63, when WB of healthy females was exposed to untreated or treated breast cancer cells. While the majority of our participants' blood showed an overall increase in platelet activation when their blood was exposed to untreated breast cancer cells, some participants showed no effect and others showed

a decrease in the percentage change of CD62P and CD63 expression (Figures 3, 5, 7 and 9). The response of platelets between participants (and/or after exposure of their WB to untreated breast cancer cells) has not been reported before although Mirlashari *et al*. (59) mentions, but doesn't show, that "no differences could be demonstrated with platelets from some "donors" but whilst in other "donors" striking differences in the kinetics of release of the two granule types were observed" when they were comparing secretion of platelet granule constituents with surface expression of the granule membrane markers CD62P and CD63.

As stated earlier, overall, the platelets of the WB samples displayed an increase in the percentage change of CD62P and CD63 expression following exposure to untreated breast cancer cells, i.e. both cells lines (MDA-MB-231: hormone-independent and MCF-7: hormone-dependent cells) were able to promote platelet activity as exhibited by the increase in the percentage change of CD62P and CD63 expression levels. These findings are in line with that reported by others. Zarà *et al*. (58) found that MDA-MB-231 and MCF-7 breast cancer cells are able to stimulate platelets to release micro particles and growth factors (vascular endothelial growth factor, transforming growth factor, platelet derived growth factor) (8) in order to initiate migration and invasion. Unfortunately the expression of platelet activation markers was not investigated by Zarà *et al*. (58) and, as a result, making a comparison is difficult. Zarà *et al*. (58) also found that there is more TF in MDA-MB-231 than in MCF-7 breast cancer cells. More recently, Pather (50, 60) found that untreated MCF-7 breast cancer cells induce a higher CD62P expression while untreated T47D breast cancer cells induce a higher CD63 expression. A further increase in CD62P and CD63 expression was found after

WB exposure to Tamoxifen-treated MCF-7 cells while a significant increase in CD62P was observed after WB exposure to Tamoxifen-treated T47D cells.

Exposing WB to MDA-MB-231 and MCF-7 breast cancer cells treated with the different seed oils induced varying levels of CD62P and CD63 expression. Overall, exposing WB to KA-, XC- and MZ- treated MDA-MB-231 breast cancer cells reduced the percentage change of CD62P expression but treating MCF-7 breast cancer cells with these oils had the opposite effect - an increase in the percentage change of CD62P expression was observed. On the whole, there was a decrease in the percentage change of CD63 expression following exposure of WB to KA-, XC- and MZ- treated MDA-MB-231 and MCF-7 breast cancer cells.

The different fatty acid profile of each seed oil could explain the differences observed (48). *K. africana* seed oil has a higher concentration of α-linolenic acid (omega-3 polyunsaturated fatty acid) while *M. zeyheri* has a higher level of linoleic acid (omega-6 polyunsaturated fatty acid) (48). In line with some of our findings, Bazán-Salinas *et al.* (61) found a decrease in platelet aggregation when subjects consumed *Vitis vinifera* (grape) or *Arachis hypogaea* (peanut) oils which contained high levels of linoleic acid and oleic acid respectively. Once again, the expression of platelet activation markers (CD62P, CD63) was not investigated by Bazán-Salinas *et al.* (61) since they used the Born turbidimetric method to determine platelet aggregation. Consequently, comparing results is difficult. Seed oils extracted from different plants contain varying concentrations of omega-3 (n-3) and omega-6 (n-6) polyunsaturated fatty acids (PUFAs). Omega-3 and omega-6 PUFAs are involved in cell membrane structure and function, cell signaling, gene expression regulation and are substrates for the

synthesis of lipid mediators (62). These PUFAs cannot be synthesized *de novo* and must be obtained from the diet (63). Omega-3 PUFAs include eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) isolated from marine sources and α-linolenic acid from leafy vegetables, nuts, and oils (62, 64). EPA and DHA can be synthesized by the conversion of α-linolenic acid *in vivo* (62). Omega-6 PUFAs include linoleic acid present in grains, meats and plant seeds (65). The exact mechanism by which these omega-3 PUFAs inhibit platelet activation has not been entirely elucidated but factors that affect membrane fluidity (66), signal transduction and thromboxane metabolism have been implicated (67). Arachidonic acid (AA), an omega-6 fatty acid, is metabolized to prostaglandin thromboxane A2 ($TxA_2$), a potent platelet activator and vasoconstrictor (67, 68). Omega-3 PUFAs suppress the conversion of AA to $TxA_2$ and act as competitors for the $TxA_2$ specific receptor (66, 67) preventing platelet activation and subsequent platelet aggregation.

Different intracellular pathways are triggered when platelets are activated by different agonists, as was observed by Taylor *et al.* (15) and Murakami *et al.* (16). When platelets were activated using platelet-activating factor (PAF), arachidonic acid (AA) or collagen, CD63 expression was greater than CD62P expression (15) and that under these conditions, there may be a preferential secretion of lysosomal content compared to α-granules. The above observations demonstrate that the activation markers expressed on the platelet surface varies with different tumor phenotypes (50, 60, 69) and/or agonists resulting in different expression of platelet activation markers.

In the present study, platelets were neither washed nor treated directly with the different seed oils; rather breast cancer cells were pre-treated with the different seed

oils prior to incubation with WB to emulate *in vivo* platelet-tumor cell interactions and probable breast tissue changes due to consumption of dietary seed oils. The effects of *in vitro* seed oil pre-treatments on breast cancer cells and the impact thereof on platelet activation have not been previously investigated in this manner.

While this preliminary study yielded promising results, a few limitations also need to be recognized. Despite substantial effect sizes in some instances, the relatively small samples sizes could have caused changes to be not statistically significant. Secondly, circulating human platelets show differences with respect to size and age (70). Platelets are extremely sensitive and if samples are not taken carefully or processed quickly, *in vitro* activation of platelets may occur resulting in erroneous conclusions (16, 17, 52, 70, 71). Future experiments should therefore consider using scanning electron microscopy to assess platelet morphology (50, 52) and/or determine platelet factor 4 and β-thromboglobulin levels (16) which are released from platelets during activation to compliment results obtained by flow cytometry.

Further experiments are also required to determine the factors which are responsible for the differential expression of the platelet activation markers, CD62P and CD63, specifically targeting pathways relating to cancer and platelet lipid metabolism.

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**Author Contributions**

MNG was responsible for conducting the research and drafted the entire manuscript. PF organized the original data and revised the manuscript. TNA was responsible for designing the study protocol and revising the manuscript. DM conducted the statistical analyses. EC revised the manuscript. WMUD provided critical revision of the manuscript.

All authors critically revised, read and approved the final manuscript.

## Declarations

**Conflicts of interest** The authors have declared no conflicts of interest with this study.

**Ethics approval** All procedures performed in studies involving human participants were in accordance with the ethical standards of the University of the Witwatersrand's Human Ethics Committee (Clearance Certificate Number M160211).

**Consent to participate** Informed consent was obtained for all subjects enrolled in this study.

## Supplementary information

Available upon request.


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## Figure Captions

**Fig. 1** Gating strategy used to detect platelet activation using unstained (a, b, d) and stained (c, e) WB. (a) A singlet population of WB was selected using forward scatter height versus forward scatter area density plot. To discriminate platelets from other cells and debris, a single platelet gate was positioned on the basis of side scatter and platelet marker (APC-conjugated mouse anti-human CD41a) (b & c). Platelet activation was then determined using FITC-conjugated mouse anti-human CD62P and phycoerythrin cyanine (PE-Cy) mouse anti-human CD63 antibodies as shown in the quadrant plot in (d) and (e)

**Fig. 2** Scatter plots showing the expression of platelet activation markers after WB was exposed to untreated and treated MDA-MB-231 breast cancer cells. (a) WB exposed to untreated cells, (b) WB exposed to cells treated with KA seed oil, (c) WB exposed to cells treated with XC seed oil and (d) WB exposed to cells treated with MZ seed oil

**Fig. 3** Graphs showing percentage change of the platelet activation marker, CD62P, of each participant, as well as the group average, after their WB was exposed to untreated (a) and treated MDA-MB-231 breast cancer cells (b, c, d). WB = WB only, MC = WB exposed to untreated breast cancer cells, KA = WB exposed to breast cancer cells treated with KA seed oil, XC = WB exposed to breast cancer cells treated with XC seed oil and MZ = WB exposed to breast cancer cells treated with MZ seed oil. P1, P2, P3 ... = Participant 1, Participant 2, Participant 3, etc. Encircled participants indicate an increase in CD62P expression after their WB was exposed to untreated MDA-MB-231 breast cancer cells

**Fig. 4** Box plot showing the pooled data of all the participants' response to the platelet activation marker CD62P to untreated and treated MDA-MB-231 breast cancer cells. WB = WB only, MC = WB exposed to untreated breast cancer cells, KA = WB exposed to breast cancer cells treated with KA seed oil, XC = WB exposed to breast cancer cells treated with XC seed oil and MZ = WB exposed to breast cancer cells treated with MZ seed oil

**Fig. 5** Graphs showing percentage change of the platelet activation marker, CD62P, of each participant, as well as the group average, after their WB was exposed to untreated (a) and treated MCF-7 breast cancer cells (b, c, d). WB = WB only, MC = WB exposed to untreated breast cancer cells, KA = WB exposed to breast cancer cells treated with KA seed oil, XC = WB exposed to breast cancer cells treated with XC seed oil and MZ = WB exposed to breast cancer cells treated with MZ seed oil. P1, P2, P3 ... = Participant 1, Participant 2, Participant 3, etc. Encircled participants indicate an increase in CD62P expression after their WB was exposed to untreated MCF-7 breast cancer cells

**Fig. 6** Box plot showing the pooled data of all the participants' response to the platelet activation marker CD62P to untreated and treated MCF-7 breast cancer cells. WB = WB only, MC = WB exposed to untreated breast cancer cells, KA = WB exposed to breast cancer cells treated with KA seed oil, XC = WB exposed to breast cancer cells treated with XC seed oil and MZ = WB exposed to breast cancer cells treated with MZ seed oil

**Fig. 7** Graphs showing percentage change of the platelet activation marker, CD63, of each participant, as well as the group average, after their WB was exposed to untreated (a) and treated MDA-MB-231 breast cancer cells (b, c, d). WB = WB only, MC = WB exposed to untreated breast cancer cells, KA = WB exposed to breast cancer cells treated with KA seed oil, XC = WB exposed to breast cancer cells treated with XC seed oil and MZ = WB exposed to breast cancer cells treated with MZ seed oil. P1, P2, P3 ... = Participant 1, Participant 2, Participant 3, etc. Encircled participants indicate an increase in CD63 expression after their WB was exposed to untreated MDA-MB-231 breast cancer cells

**Fig. 8** Box plot showing the pooled data of all the participants' response to the platelet activation marker CD63 to untreated and treated MDA-MB-231 breast cancer cells. WB = WB only, MC = WB exposed to untreated breast cancer cells, KA = WB exposed to breast cancer cells treated with KA seed oil, XC = WB exposed to breast cancer cells treated with XC seed oil and MZ = WB exposed to breast cancer cells treated with MZ seed oil

**Fig. 9** Graphs showing percentage change of the platelet activation marker, CD63, of each participant, as well as the group average, after their WB was exposed to untreated (a) and treated MCF-7 breast cancer cells (b, c, d). WB = WB only, MC = WB exposed to untreated breast cancer cells, KA = WB exposed to breast cancer cells treated with KA seed oil, XC = WB exposed to breast cancer cells treated with XC seed oil and MZ = WB exposed to breast cancer cells treated with MZ seed oil. P1, P2, P3 ... = Participant 1, Participant 2, Participant 3, etc. Encircled participants indicate an increase in CD63 expression after their WB was exposed to untreated MCF-7 breast cancer cells

**Fig. 10** Box plot showing the pooled data of all the participants' response to the platelet activation marker CD63 to untreated and treated MCF-7 breast cancer cells. WB = WB only, MC = WB exposed

to untreated breast cancer cells, KA = WB exposed to breast cancer cells treated with KA seed oil, XC = WB exposed to breast cancer cells treated with XC seed oil and MZ = WB exposed to breast cancer cells treated with MZ seed oil