Indonesian Journal of Biotechnology

VOLUME 30(1), 2025, 47-55 | RESEARCH ARTICLE

Black seed oil inhibits the migration of triple-negative breast cancer cells and regulates MMP-9 expression

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SUBMITTED 16 May 2024 **REVISED** 27 February 2025 **ACCEPTED** 4 March 2025

ABSTRACT Black seed (*Nigella sativa* L.) is well known for its pharmacological properties, particularly its anticancer activity, with previous studies demonstrating its cytotoxic effects on several cell lines, such as A-549, DLD-1, MDA-MB231, or HCT. This study aims to investigate the effects of black seed oil (BSO) on the migratory activity of 4T1 triple-negative breast cancer (TNBC) cells, focusing on its bioactive properties. BSO was extracted via hydro-distillation and analyzed for its phytochemical composition using gas chromatography–mass spectrometry (GC-MS). The cytotoxicity of BSO and doxorubicin (Dox) was assessed using the MTT assay. The effects of BSO and Dox on cell migration and matrix metalloproteinase-9 (MMP-9) expression were evaluated using a scratch wound-healing assay and gelatin zymography method respectively. Additionally, intracellular reactive oxygen species (ROS) levels were measured using 2',7'-dichlorofluorescin diacetate (DCFDA) staining. GC-MS analysis identified p-cymene as a major component of BSO, along with various other bioactive compounds. BSO exhibited low toxicity toward 4T1 cells, while its combination with Dox reduced cell viability in a dose-dependent manner. Furthermore, BSO in combination with Dox inhibited cell migration and suppressed MMP-9 expressions in 4T1 cells. BSO treatment also led to an increase in ROS levels. In conclusion, BSO exhibits potential anticancer properties by inhibiting cell migration and downregulating MMP-9 expression, highlighting its possible therapeutic role in TNBC treatment.

KEYWORDS 4T1; Migration; MMP-9; *Nigella sativa*; Triple-negative breast cancer

1. Introduction

Reactive oxygen species (ROS) play a critical role in cancer progression, acting as both a driver of tumor survival and a potential therapeutic target. Cancer cells exhibit elevated ROS levels due to genetic mutations, metabolic reprogramming, and the influence of the tumor microenvironment (Kumari et al. 2018). While moderate ROS levels promote cell proliferation and survival, excessive ROS accumulation leads to oxidative stress, resulting in apoptosis or necrosis (Larasati et al. 2018). One of the key mechanisms by which ROS influence cancer progression is through the mitogen-activated protein kinase (MAPK) pathway, which is involved in cell proliferation, survival, and stress responses. ROS-mediated activation of MAPK pathways, including ERK1/2, JNK, and p38, contributes to tumor growth and resistance to therapy (Reczek and Chandel 2017). Additionally, ROS regulate matrix metalloproteinase (MMP) expression, particularly MMP-9, which facilitates extracellular matrix degradation, promoting cancer cell invasion and metastasis (Mori et al. 2019).

Chemotherapy induces oxidative stress in cancer cells by increasing ROS levels beyond their tolerance threshold, leading to cell death. However, in some cases, cancer cells adapt to oxidative stress by activating survival pathways, including MAPK and Phosphoinositide 3-kinase (PI3K)/Akt, which contribute to chemotherapy resistance (Karagiannis et al. 2018). The upregulation of MMP-9 in response to chemotherapy further enhances tumor aggressiveness by facilitating metastasis (Kumari et al. 2018). Consequently, targeting ROS, MAPK, and MMP-9 in combination therapy could enhance chemotherapy efficacy while reducing resistance. Natural compounds with antioxidant and pro-oxidant properties have been explored as adjuvants in chemotherapy, helping to modulate ROS levels and suppress MMP expression (Mileo and Miccadei 2016). By integrating ROS-targeting strategies with chemotherapy, it may be possible to disrupt tumor survival mechanisms, enhance apoptosis, and prevent metastasis, ultimately improving treatment outcomes in aggressive cancers such as TNBC.

Black seed is a well-studied herb with numerous known pharmacological effects (Islam et al. 2019; Mollazadeh et al. 2017; Mukhtar et al. 2019; Shokri 2016),

and it is considered a potential chemoprevention agent that can reduce the toxicity of chemotherapy, including Doxorubicin (Dox) (Hosseinzadeh et al. 2018; Usmani et al. 2019). The pharmacological activity of black seed is attributed to its phytochemical content, rich in volatile and non-volatile bioactive compounds, including, monoterpenes and sesquiterpenes, such as p-cymene, thymoquinone, α-thujene, longifolene, carvacrol, and longipinene (Mohammed et al. 2019; Ali et al. 2022; Mehraj et al. 2022). Aqueous and ethanol extracts of the black seed primarily contain polyphenols, flavonoids, and alkaloids, which may have different pharmacokinetics and therapeutic effects than black seed oil (BSO). The lipophilic nature of the BSO can enhance its ability to penetrate cancer cell membranes, increasing its bioavailability and intracellular uptake. Thymoquinone and p-cymene, the hydrophobic constituents of BSO, are known to modulate oxidative stress and redox signaling pathways, including ROS generation, MAPK activation, and MMP inhibition, all of which are crucial targets in cancer therapy (Mahmoudvand et al. 2014).

This study provides novel insights into the anticancer potential of BSO in combination with Dox by demonstrating its ability to inhibit migration (wound healing assay) and suppress MMP-9 expression (gelatin zymography assay) in triple-negative breast cancer (TNBC) 4T1 cells. Unlike previous research focusing on cytotoxic effects in other cancer cell lines, this study highlights BSO's anti-metastatic properties and its role in modulating ROS levels, suggesting a potential pro-oxidant effect in cancer cells. Additionally, the findings reveal a synergistic effect between BSO and Dox, reducing cell viability in a dose-dependent manner, which may enhance chemotherapy effectiveness. These results underscore BSO's potential as a therapeutic agent for TNBC, particularly in preventing metastasis.

2. Materials and Methods

2.1. Extraction process of black seeds (Nigella sativa L.)

The *Nigella sativa* L. seeds were obtained from the Materia Medica Batu Malang, which also provided authentication of the herbal samples (reference number 074/055/102.7/2017, UPT. Materia Medica Batu, Malang, Indonesia). The seeds were crushed and subjected to hydro-distillation using a Clevenger apparatus system to isolate BSO, following the method described by (Benkaci-Ali et al. 2007) with slight modifications. Hydro-distillation of black seeds (574 g) was performed with water (3 L) for 3 h, yielding 0.31 g of yellow aromatic oil. To remove residual water, sodium sulfate was added to the isolated oil and left for 24 h. The dried oil was then stored in the dark at 4 °C (Aliyah et al. 2021).

2.2. Phytochemical composition analysis of BSO using GC-MS

BSO was analyzed using a SHIMADZU gas chromatograph coupled with a QP2010S mass spectrometer. Helium was used as the carrier gas in an Rtx-5 MS fused silica capillary column with the following specifications, i.e. length of 30 m, inner diameter of 0.25 mm, and film thickness of 0.25 µm. The GC operating parameters included a column oven temperature of 70 °C, an injection temperature of 300 °C, a pressure of 13.7 kPa, and a solvent cut time of 3 min. The injector operated in split mode with a split ratio of 1:49. The column temperature was initially set at 70 °C and gradually increased to 300 °C. Mass spectrometry (MS) scanning was conducted within the mass-to-charge ratio (m/z) range of 28 to 600, starting from 3.20 to 70 min. Compound identification was performed by comparing mass spectra with standard libraries (WileyMS and NIST Libraries version 2019) based on relative retention times (Aliyah et al. 2021).

2.3. 4T1 cells culture

The 4T1 breast cancer cells were acquired from Prof. Masashi Kawaichi, M.D., Ph.D., at the Nara Institute of Science and Technology (NAIST), Japan. The cells were cultured in high-glucose Dulbecco's Modified Eagles Medium (DMEM) (Sigma, St. Louis, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma), HEPES, sodium bicarbonate, 0.5 µg/mL Fungizone, 1000 U/mL of Penicilin and 1000 U/mL of Streptomycin (Gibco, New York, USA). The cells were maintained in an incubator at 37 °C under a 5% CO_2 atmosphere (Ahlina et al. 2020).

2.4. Cytotoxic assay

The 4T1 breast cancer cells were seeded (approximately 2.5×10^3 cells/well) in 96-well plates and incubated in a controlled environment for 24 h. The BSO stock solution was first prepared by dissolving BSO in dimethyl sulfoxide (DMSO). This stock solution was further diluted to obtain the desired BSO treatment concentrations. Throughout the cell culture treatment, the final DMSO concentration was maintained at a maximum of 0.1% (v/v) to minimize potential cytotoxic effects. The cells were exposed to varying concentrations of BSO (1, 5, 25, 50, 100, 150, and 200 µg/mL) for a duration of 24 h. The medium was removed and the cells were rinsed with 1× phosphate-buffered saline (PBS). A solution of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Thermo Fisher Scientific) at a concentration of 0.5 mg/mL was added to the medium and incubated for a period of 2–3 h. Each well was treated with a 10% SDS solution to halt the MTT reaction, and the plate was then incubated overnight in a dark room. A Bio-rad microplate reader was utilized to quantify absorbance at a wavelength of 595 nm. The experiment was conducted in triplicate, and the absorbance was measured to determine cell viability relative to untreated

samples. Cell viability was determined by combining BSO at specific doses (50 and 100 µg/mL) with a low concentration of Dox (10 nM) (Aliyah et al. 2021).

2.5. Determination of intracellular ROS levels using DCFDA staining

Triple-negative breast cancer cells, 4T1, were cultured in 24-well plates at a density of 7×10^4 cells/well and incubated for 24 h. Following incubation, the culture medium was removed, and the cells were washed with 1× PBS. Cell detachment was performed using a 0.25% trypsin-EDTA solution and a supplemented buffer. The collected cells were then transferred to opaque microtubes and labeled with 25 µM of 2',7'-dichlorofluorescin diacetate (DCFDA). The labeling process was carried out by incubating the cells at 37 °C for 30 min. Subsequently, the cells were treated with BSO (100 and 200 µg/mL) in combination with Dox (100 nM) for 4 h. The intracellular ROS levels were then analyzed using flow cytometry, with an excitation wavelength of 485 nm and an emission wavelength of 535 nm (Ahlina et al. 2020).

2.6. Wound healing assay

Wound healing assays are widely used to assess cell migration, a critical process in cancer metastasis. By creating an artificial wound in a cell monolayer, this assay allows researchers to evaluate the ability of cancer cells to migrate and close the wound, providing insights into the invasive potential of tumor cells. The wound healing assay was performed according to a previously established protocol. The 4T1 cells were seeded in a 24-well plate at a density of approximately 8.7×10^4 cells per 500 µL and incubated at 37 °C for 24 h until reached 80% confluence. The cells were washed and treated with a 0.5% FBS solution for 24 h to induce a quiescent state. A scratch was created in the cell monolayer using a sterile scratcher, after which the cells were rinsed and treated with varying concentrations of BSO (50 and 100 µg/mL) and Dox (10 nM). The cultures were maintained at 37 °C for 48 h, and cell migration was observed and documented at 0, 18, 24, and 42 h using an inverted microscope (Nurrachma et al. 2020).

2.7. Gelatin zymography assay

Gelatin zymography is a sensitive enzymatic technique used to analyze the activity of matrix metalloproteinases (MMPs), particularly MMP-9, which plays a significant role in extracellular matrix degradation and cancer cell invasion. Since MMP-9 facilitates tumor progression by promoting metastasis, assessing its expression and activity is crucial for understanding the mechanisms by which BSO influences cancer cell invasiveness. By combining the wound healing assay with gelatin zymography, this study provides comprehensive insights into the potential anti-metastatic effects of BSO through its regulation of cell migration and MMP-9 activity (Toth et al. 2012). The cells were seeded in a 6-well plate at a density of 2×10^5 cells per well in 1 mL of culture medium and incubated at 37 °C for 24 h. The following day, the medium was replaced with a medium containing 0.5% FBS and incubated for an additional 24 h. The cells were rinsed with 1 mL PBS)and treated with BSO at concentrations of 50 and 100 µg/mL. A 10 nM concentration of Dox was used as a positive control to induce MMP-9 production. The cells were further incubated for 24 h, and the culture medium was collected and centrifuged at 4°C to obtain the supernatant. The collected supernatant was mixed with a loading buffer and subjected to electrophoresis for 130 min at 110 V and 50 A. After electrophoresis, the gel was immersed in a renaturation solution containing Triton-X 100 for 30 min to remove SDS. The gel was then incubated in a specific incubation buffer at 37 °C for 20 h. After incubation, the gel was stained with Coomassie Brilliant Blue (CBB-G-250, Sigma Aldrich, Germany) for 30 min and subsequently destained until a clear transparent blue band became visible, indicating MMP-9 activity (Nurrachma et al. 2020).

2.8. Statistical analysis

All data were analyzed using Microsoft excel. The scratched was quantified at various time by using Image J software and the percent closure area recorded. Percent closure was analyzed using t-test ($p < 0.05$).

3. Results and Discussion

3.1. Phytochemical identification of BSO with GC-MS

The chromatogram of BSO revealed 13 compounds (Table 1, Figure 1). The compounds in BSO corresponding to major peaks confirmed as p-cymene (48.03%), dihydrocarveol (11.39%), α-tujen (11.29%), limonene (5.43%), β-pinen (4.55%), α-pinene (3.03%).

3.2. Cytotoxic activity of BSO in 4T1 breast cancer cells

In this study, a single treatment of BSO on 4T1 cells used concentration range of 1–200 µg/mL. BSO-treated cells showed lower cell viability compared to Dox. No IC_{50} value was found from BSO treatment on 4T1 cells up to concentration of 200 µg/mL. A cytotoxicity profile was needed to define the non-cytotoxic concentration to observe migration activity. We used a low dose of Dox, in which 10 nM, to increase migratory effect. Moreover, the effect of combination treatment with BSO (50 and 100 µg/mL) and Dox 10 nM on cell viability was also ensured (Figure 2). The combination of BSO and Dox significantly decreased the cell viability compared to single treatment of Dox.

3.3. BSO and Dox-combination elevated intracellular ROS level in 4T1 breast cancer cells

The effect of combination of BSO 100 and 200 µg/mL with 100 nM Dox were defined as their effect on level of intracellular ROS. We used Dox 100 nM as an inducer of ROS. The results showed that compared to Dox, single treatment of BSO induced elevation of ROS in a dose dependent manner (Figure 3). Additionally, combined treatment of Dox and 200 ug/mL of BSO increased the intra-

TABLE 1 Phytochemical constituent of BSO from GC-MS analysis.

Peak Number	Retention time (min)	Chemical Formula	Compound Identification	% of Abundance
1	9.725	$C_{10}H_{16}$	α-Thujene	11.29
2	9.983	$C_{10}H_{16}$	α-pinene	3.03
3	11.5	$C_{10}H_{16}$	Sabinene	1.81
4	11.675	$C_{10}H_{16}$	β-pinene	4.55
5	13.167	$C_{10}H_{16}$	α-terpinolene	0.57
6	13.542	$C_{10}H_{14}$	p-cymene	48.03
7	13.65	$C_{10}H_{16}$	Limonene	5.43
8	14.683	$C_{10}H_{16}$	γ-terpinene	2.48
9	15.992	$C_{10}H_{16}O_2$	Nonanoic Acid	2.02
10	16.808	$C_{10}H_{18}O$	Dihydrocarveol	11.39
11	18.983	$C_{10}H_{18}O$	Terpinen-4-ol	0.81
12	22.15	$C_{12}H_{20}O_2$	Bornyl acetate	0.31
13	23.983	$C_{15}H_{24}$	Naphthalene	0.38
14	24.15	$C_{15}H_{24}$	α-longipinene	1.34
15	24.858	$C_{15}H_{24}$	α-copaene	0.85
16	25.892	$C_{15}H_{24}$	Junipene	4.98
17	26.125	$C_{15}H_{24}$	β-caryophyllene	0.74

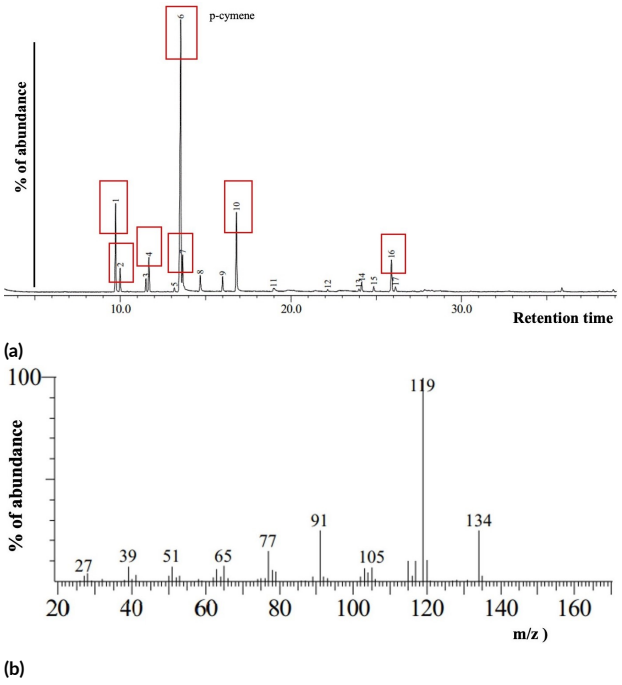

FIGURE 1 Phytochemical Identification of BSO using GC-MS. The gas chromatography-mass spectrometry (GC-MS) analysis was performed as described in the methods section. (A) The gas chromatogram of BSO revealed 17 peaks, corresponding to 13 identified compounds. The most abundant component was peak number 6, identified as p-cymene. Other prominent peaks corresponded to α-thujene (1), α-pinene (2), β-pinene (4), limonene (7), dihydrocarveol (10), and Junipene (16). (B) The mass spectrum of p-cymene detected in BSO is presented, with a molecular weight of 134 g/mol, as determined from the mass spectrogram.

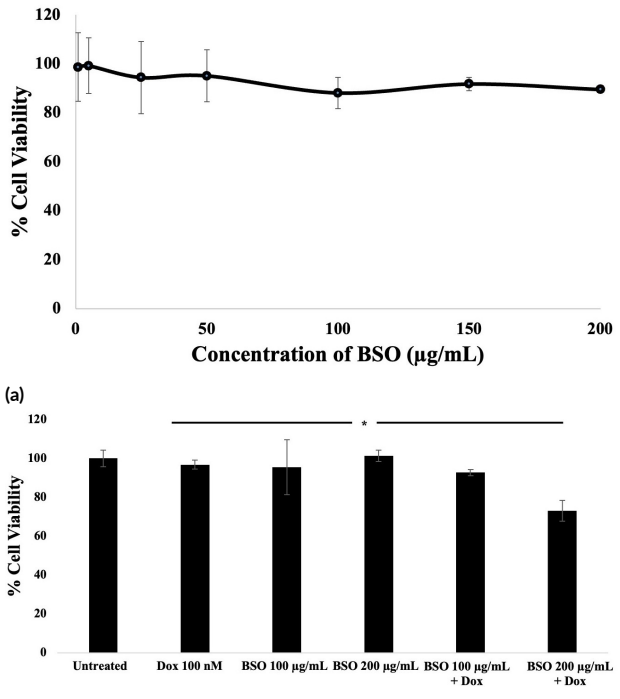

FIGURE 2 Cytotoxic Effects of BSO and Its Co-Chemopreventive Potential in Combination with Doxorubicin on 4T1 Cells. The cytotoxicity of BSO and its combination with Dox on 4T1 cells was evaluated using the MTT assay after 24 hours of treatment. (A) The cell viability profile of BSO, presented as mean ± SD from three independent experiments, indicates that BSO exhibited low cytotoxic effect toward 4T1 cells, with an IC_{50} value exceeding 200 µg/mL. (B) The cytotoxic effect of BSO in combination with Dox (10 nM) was assessed, where BSO at concentrations of 100 and 200 µg/mL, in combination with 10 nM Dox, significantly reduced cell viability compared to other treatment groups (Student's t-test, $^*p < 0.05$).

cellular levels of ROS.

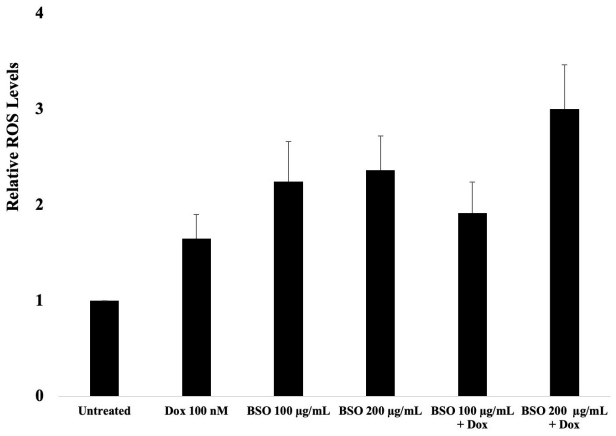

FIGURE 3 BSO Increases Intracellular ROS Levels on 4T1 Cells. Intracellular reactive oxygen species (ROS) levels were measured using 2′,7′-dichlorofluorescin diacetate (DCFDA) staining followed by flow cytometry analysis in 4T1 breast cancer cells after 24h of BSO and Dox treatment. This assay was conducted to evaluate the effects of BSO alone and in combination with Dox on ROS generation. A single treatment with BSO significantly increased intracellular ROS levels compared to the untreated control group (Student's t-test, $^*p < 0.05$). Furthermore, the combination of BSO 200 µg/mL with Dox induced a greater increase in intracellular ROS levels compared to Dox single treatment.

3.4. Antimigration properties of BSO and Dox-combination in 4T1 breast cancer cells

This study aimed to elucidate the anti-migration effect of BSO on TNBC using 4T1 cells. Previously, BSO concentration at less than 200 µg/mL did not have a significant impact on cell viability. To this end, we used 50 and 100 µg/mL to observe the migration activity of 4T1 cells. Untreated cells migrated along the edge of the wound and covered in 48 h. A low dose of Dox promoted migratory activity in comparison with untreated cells, but the difference was not significant. Combined BSO 100 µg/mL and Dox inhibit migration on 4T1 cells.

3.5. BSO and Dox-combination decreased MMP-9 expressions in 4T1 breast cancer cells

Migration in cancer cells is regulated by various kinds of protein, including matrix metalloproteinase (MMPs), which can facilitate cell migration by degrading the extracellular matrix and induce adhesion removal and cell cleavage (Jabłońska-Trypuć et al. 2016). For this reason, the gelatin zymography assay will clarify the possibility of migration activity regulated by MMP, especially MMP-9. Single treatment of BSO at 50 and 100 µg/mL increased MMP-9 expression. Combination of Dox 10 nM and BSO 50 and 100 µg/mL decreased MMP-9 expression in a dose dependent manner (Figure 5).

3.6. Discussion

This study provides novel insights into phytochemicals constituent of BSO, and the anticancer potential of BSO by demonstrating its ability to increased ROS levels, to inhibit cell migration and to suppress MMP-9 expression in 4T1 cells, a highly aggressive cancer subtype. The characterization of BSO obtained through the distillation process was conducted using GC-MS, which is suitable for analyzing the phytochemical composition of BSO, as its constituents primarily consist of oils, fats, and volatile ter-

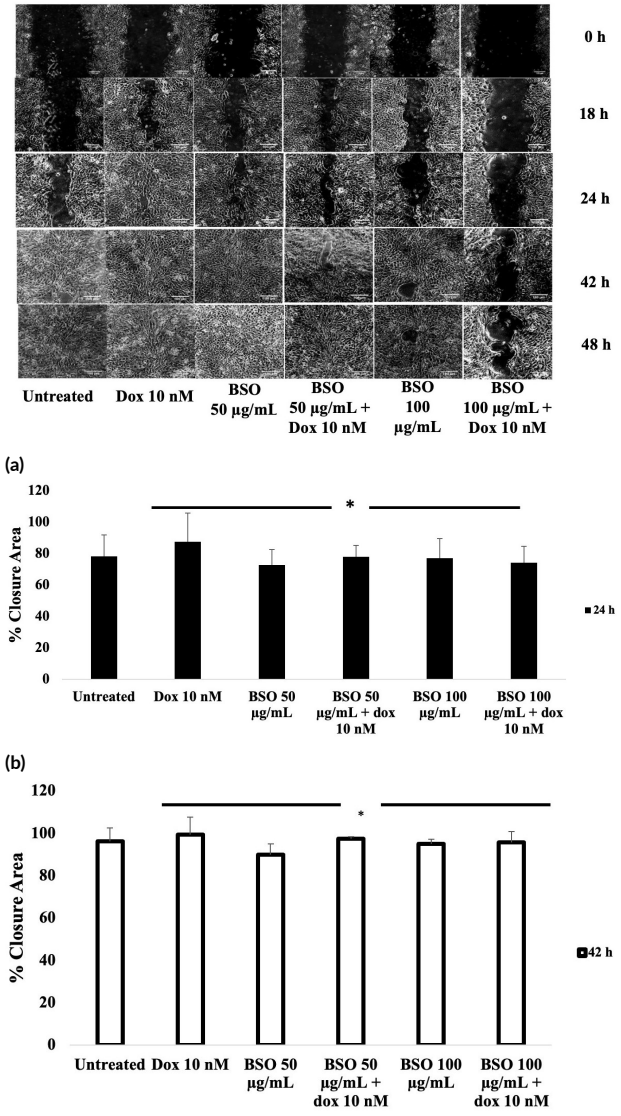

FIGURE 4 Anti-Migratory Effect of BSO and Its Combination with Doxorubicin on 4T1 Cells. The scratch wound healing assay was conducted to assess the anti-migratory potential of BSO, Dox, and their combination following 42 hours of BSO treatment. (A) Representative images show cell morphology after scratch introduction and treatment with BSO at concentrations of 100 and 200 µg/mL, while Dox was used at 10 nM. Observations were made at 18, 24, and 42 hours post-treatment using an inverted microscope at 100× magnification. (B) The percentage of wound closure was quantified to evaluate the migration-inhibitory effects of BSO and Dox, either as single treatments or in combination after 24h of drugs treatment. (C) The percentage of wound closure was quantified to evaluate the migration-inhibitory effects of BSO and Dox, either as single treatments or in combination after 42 h of drugs treatment. The scratch area was analyzed using ImageJ software, and the percentage of wound closure was calculated accordingly. BSO at 100 µg/mL, when combined with 10 nM Dox, showed a statistically significant reduction in cell migration compared to other treatment groups ($^*p < 0.05$, Student's t-test).

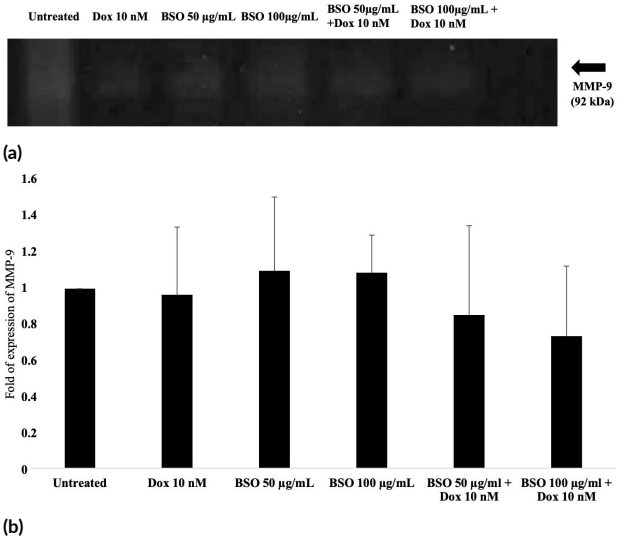

FIGURE 5 The Effect of BSO–Dox Combination on the Suppression of MMP-9 Expression in 4T1 Cells. The 4T1 cells were seeded at a density of approximately 2×10^5 cells and incubated for 24 hours prior to treatment with BSO and doxorubicin (Dox) for an additional 24 hours. Following treatment, the culture medium was collected for gelatin zymography analysis. (A) The band profile of MMP-9 expression was visualized through gelatin zymography. Band intensities were quantified using ImageJ software (n = 3). (B) The quantification of MMP-9 band intensity, expressed as a percentage, was analyzed using a Student's t-test ($p < 0.05$). The combination treatment of BSO and Dox significantly reduced MMP-9 expression levels compared to Dox (10 nM) treatment alone.

penes found in essential oils (Kazemi 2014).

The GC-MS results revealed that the distilled BSO contained a total of 17 compounds, as indicated by the presence of 17 peaks (Figure 1, and Table 1). Interestingly, the analysis showed that the BSO did not contain thymoquinone, a bioactive compound extensively researched for its potential anticancer properties. This finding contrasts with previous studies, which reported that BSO from Iran, extracted using petroleum ether, had a significantly higher thymoquinone content (42%) (Mahmoudvand et al. 2014). Similarly, Burits and Bucar (2000) found that BSO extracted through Soxhlet extraction with petroleum ether as the solvent contained 30%–48% of thymoquinone. Another research from Tunisia, indicated that BSO extracted via hydro distillation, contained only a minimal amount of thymoquinone (0.79%) (Harzallah 2012). This study found that the BSO from UPT Materia Medika Malang has a higher concentration of p-cymene, indicated by the prominent peak at the sixth position in the chromatogram (Figure 1). Mass spectrometry analysis identified predicted chemical compounds present in the BSO, while the gas chromatogram (Figure 1) displayed several peaks, with some being more prominent than others. These peaks represent the percentage of peak area, which correlates with the relative abundance of each compound detected in the sample. Based on the Wiley229 Library, peak number 6 was identified as p-cymene (Figure 1a). The distilled black seed oil primarily contained hydrocarbon terpenoids, including α-pinene, β-pinene, sabinene, α-terpinene, limonene, γ-terpinene, p-cymene, and terpinolene, as well as several sesquiterpene compounds such as α-longipinene and junipene.

In this study, p-Cymene, a natural monoterpene commonly found in essential oils of various medicinal plants, including N. sativa, is the most abundance of phytochemical compounds in BSO samples, and it has been studied for its diverse pharmacological properties, including as anticancer. Several studies have reported that p-cymene exhibits antiproliferative, pro-apoptotic, and anti-metastatic activities in different cancer cell lines. p-cymene has been reported to reduce cancer cell migration and invasion, particularly in aggressive cancers. Moreover, p-cymene inhibits the expression and activity of matrix metalloproteinases (MMPs), including MMP-2 and MMP-9, which are crucial enzymes for tumor invasion and metastasis (Balahbib et al. 2021). α-pinene induce G2/M phase cell cycle arrest and increased ROS generation in A549 lung cancer cells (Xu et al. 2018). Meanwhile, β-Pinene exhibited strong cytotoxic activity against melanoma (B16F10) and breast cancer (MCF-7) cells (De Lima et al. 2014), increases ROS levels, triggering oxidative stress-mediated cancer cell death (Bicas et al. 2011). A study on lung cancer cells showed that α-terpinene inhibited cell migration and invasion by downregulating MMP-2 and MMP-9 (Kang et al. 2016). These monoterpenes—α-pinene, β-pinene, sabinene, α-terpinene, and limonene—exhibit strong anticancer activities through multiple mechanisms, including apoptosis induction, inhibition of cancer proliferation, suppression of metastasis, ROS generation, and enhancement of chemotherapy sensitivity.

This study showed that BSO did not have cytotoxicity effect on 4T1 cells up to concentration of 200 µg/mL. The BSO has been extensively studied for its cytotoxic effects against several cancer cells, including human lung cancer cells (A549 cells) (Al-Sheddi et al. 2014); colorectal cancer cells (HCT cells) (Al-Rajhi et al. 2024); the triple negative breast cancer cells, MDA-MB 231 cells (Ma and Peng 2024), and MCF-7 breast cancer cells (Çınar et al. 2024). Additionally, the findings reveal a synergistic effect between BSO and Dox, reducing cell viability in a dose-dependent manner, which may enhance chemotherapy effectiveness. Consequently, 4T1 cells exhibit distinctive traits, specifically significant migratory capacity, which BSO may effectively target; additional investigation is warranted, potentially employing elevated BSO concentrations.

This study showed that combined Dox and BSO increased ROS levels on 4T1 cells. These findings underscored the potential of BSO as a combination therapeutic agent for TNBC. It is known that ROS is involved in cellular responses mediated by integrin, in which elevation of ROS levels occurred when integrin binds to an antibody or matrix enzyme (Zeller et al. 2013). This phenomenon will trigger activation of oxidases, such as NOX, which promotes invadopodium formation, and membrane protrusion for invasion (Vermot et al. 2021). On the other hand, ROS is one of parameters for cancer therapy, in which some

chemotherapy drugs have been known to elevate the intracellular levels of ROS, such as Dox, by forming complexes with topoisomerase to create double-strand breaks, and induce apoptosis (Zhu et al. 2016). The differences in ROS function in cell progression or antitumor activity are due to differences cancer stage, development, and progression (Liao et al. 2019). Therefore, the potential combinational chemotherapeutic of BSO should be further explored for their capacity to increase ROS generation.

The results of this study showed that combined Dox and BSO inhibited migration on 4T1 cells. Previously, black seed have been investigated and their saponin content revealed to suppress activator protein-1 (AP-1) in colorectal carcinoma cells which correlates with promotion, progression, and metastasis of cancer cells (Elkady et al. 2015). One of the BSO component, thymoquinone is known to inhibit metastasis in renal cancer cells through autophagy (Zhang et al. 2018). BSO is proven to inhibit the migration of 4T1 cells at selected concentrations (Figure 4) compared with the single Dox treatment. This concentration was determined from a single cytotoxic result, which illustrates that the concentration of 200 µg/mL BSO did not further reduce the cell population (Figure 2). A low concentration in the migration assay is important to reduce the cytotoxic effect on migration activity (Ramadani et al. 2018). In the future, further research is required to reveal the anti-migratory activity of BSO at higher concentrations.

This study showed that combined Dox and BSO decreased expression of MMP-9 on 4T1 cells. Before migration occurs, cells need to invade the stroma by using protease enzymes to degrade the extracellular matrix (Wisdom et al. 2018). Examples of these enzymes are MMP, cathepsins, and urokinase plasminogen activator (uPAR) (Gautam et al. 2018). Therefore, to clarify the proteins that play a role in migration activity, the gelatin zymography is needed to identify expression of MMP-9. Treatment with BSO was able to reduce MMP-9 expression. This clarifies that BSO inhibits the migratory activity of 4T1 cells via MMP-9 regulation.

Piskounova et al. (2015) declared that ROS elevation was related to the maintenance of the cell metastasis phenotype, in contrast to the result of this study, which showed that high levels of ROS may trigger cell death and affect cell progression especially cancer metastasis. The interesting phenomenon observed in this study was in line to the result of Larasati et al. (2018) who found that elevation of ROS above a threshold will trigger cells into the senescence and apoptosis. In addition, a previous review by Marioli-Sapsakou and Kourti (2021) stated that ROS can be a target for cancer therapy by increasing ROS levels to high or excessive level. Thus, this statement strengthens the results of our research that BSO combined with an elevation level of ROS can inhibit the progression of 4T1 breast cancer cells through migration and MMP-9 regulation.

This study found that p-Cymene was a major phytochemical compound in BSO samples. In addition, BSO showed no cytotoxicity effect on 4T1 cells at a concentration of 200 µg/mL. However, when combined with Dox, BSO increased ROS levels on 4T1 cells, suggesting its potential as a combination therapeutic agent for TNBC. The study also found that combined Dox and BSO inhibited migration and decreased MMP-9 expression on 4T1 cells. The possible combinatorial chemotherapeutic effects of BSO should be further investigated in for the elucidation of their molecular mechanisms.

4. Conclusions

This study demonstrated that BSO exhibits anti-migration effects in Dox-treated 4T1 breast cancer cells by inhibiting MMP-9 expression, as observed in the gelatin zymography assay. Furthermore, BSO significantly enhances ROS generation, which serves as a key marker for oxidative stress-induced cancer suppression. The increase in ROS levels correlates with reduced metastatic potential, highlighting BSO's potential as a targeted therapy for aggressive breast cancers. These findings suggest that BSO, either alone or in combination with chemotherapeutic agents, Dox, could serve as a promising adjuvant strategy in limiting breast cancer progression. Further studies are needed to explore the molecular mechanisms related the findings of this study.

Acknowledgments

We thank to Recognition Final Project from Universitas Gadjah Mada who support this research with contract serial number 2129/UN1/DITLIT/DIT-LIT/LT/2019.

Authors' contributions

GL contributed to the acquisition and interpretation of data and drafting of the article. AAN and GGM contributed to the acquisition and interpretation of data. AH contributed to the conception and design of the study, funding acquisition, supervision, interpretation of data, revising the article, and final approval of the version to be published. EM contributed to the conception and design of the study, interpretation of data, and supervision.

Competing interests

The authors have no conflict of interest to declare.

References

Ahlina FN, Nugraheni N, Salsabila IA, Haryanti S, Da'i M, Meiyanto E. 2020. Revealing the reversal effect of galangal (*Alpinia galanga* L.) extract against oxidative stress in metastatic breast cancer cells and normal fibroblast cells intended as a Co-chemotherapeutic and anti-ageing agent. Asian Pacific J. Cancer Prev. 21(1):107–117. doi:10.31557/APJCP.2020.21.1.107.

Al-Rajhi A, Abdelghany T, Almuhayawi M, Alruhaili M, Saddiq A, Baghdadi A, AL Jaouni S, Albasri H, Waznah M, Alraddadi F, Selim S. 2024. Effect of ozonation on the phytochemicals of black seed oil and its anti-microbial, anti-oxidant, anti-inflammatory, and anti-neoplastic activities *in vitro*. Sci. Rep. 14:30445. doi:10.1038/s41598-024-81157-9.

Al-Sheddi ES, Farshori NN, Al-Oqail MM, Musarrat J, Al-Khedhairy AA, Siddiqui MA. 2014. Cytotoxicity of *Nigella sativa* seed oil and extract against human lung cancer cell line. Asian Pacific J. Cancer Prev. 15(2):983–987. doi:10.7314/APJCP.2014.15.2.983.

Ali SR, Hussain ST, Ahsan SA, Shehzadi N, Ashfaq M, Bano S, Akhlaq M, Umrani JH. 2022. Black Seeds (*Nigella sativa* L.) -kalonji: A brief review of its anticancer and anti- Tumour qualities. Hamdard Med. 65(1):25–33.

Aliyah AN, Lintangsari G, Maran GG, Hermawan A, Meiyanto E. 2021. Cinnamon oil as a co-chemotherapy agent through inhibition of cell migration and MMP-9 expression on 4T1 cells. J. Complement. Integr. Med. 19(4):921–928. doi:10.1515/jcim-2020-0165.

Balahbib A, El Omari N, Hachlafi NE, Lakhdar F, El Menyiy N, Salhi N, Mrabti HN, Bakrim S, Zengin G, Bouyahya A. 2021. Health beneficial and pharmacological properties of p-cymene. Food Chem. Toxicol. 153:112259. doi:10.1016/j.fct.2021.112259.

Benkaci-Ali F, Baaliouamer A, Meklati BY, Chemat F. 2007. Chemical composition of seed essential oils from Algerian *Nigella sativa* extracted by microwave and hydrodistillation. Flavour Fragr. J. 22(2):148–153. doi:10.1002/ffj.1773.

Bicas JL, Neri-Numa IA, Ruiz AL, De Carvalho JE, Pastore GM. 2011. Evaluation of the antioxidant and antiproliferative potential of bioflavors. Food Chem. Toxicol. 49(7):1610–1615. doi:10.1016/j.fct.2011.04.012.

Burits M, Bucar F. 2000. Antioxidant activity of *Nigella sativa* essential oil. Phyther. Res. 14(5):323–328. doi:10.1002/1099-1573(200008)14:5<323::AID-PTR621>3.0.CO;2-Q.

De Lima VT, Vieira MC, Kassuya CA, Cardoso CA, Alves JM, Foglio MA, De Carvalho JE, Formagio AS. 2014. Chemical composition and free radical-scavenging, anticancer and anti-inflammatory activities of the essential oil from *Ocimum kilimandscharicum*. Phytomedicine 21(11):1298–1302. doi:10.1016/j.phymed.2014.07.004.

Elkady AI, Hussein RA, El-Assouli SM. 2015. Mechanism of action of *Nigella sativa* on human colon cancer cells: The suppression of AP-1 and NF-κB transcription factors and the induction of cytoprotective genes. Asian Pacific J. Cancer Prev. 16(17):7943–7957. doi:10.7314/APJCP.2015.16.17.7943.

Gautam J, Banskota S, Lee H, Lee YJ, Jeon YH, Kim JA, Jeong BS. 2018. Down-regulation of cathepsin S and matrix metalloproteinase-9 via Src, a non-receptor tyrosine kinase, suppresses triple-negative breast cancer growth and metastasis. Exp. Mol. Med. 50(9):1–14. doi:10.1038/s12276-018-0135-9.

Harzallah HJ. 2012. Chemical composition, antibacterial and antifungal properties of Tunisian *Nigella sativa* fixed oil. African J. Microbiol. Res. 6(22):4675–4679. doi:10.5897/ajmr11.1073.

Hosseinzadeh L, Soheili S, Ghiasvand N, Ahmadi F, Shokoohinia Y. 2018. Fatty acid mixtures from *Nigella sativa* protects PC12 cells from oxidative stress and apoptosis induced by doxorubicin. Pharm. Sci. 24(1):15–22. doi:10.15171/PS.2018.04.

Islam MT, Khan MR, Mishra SK. 2019. An updated literature-based review: phytochemistry, pharmacology and therapeutic promises of *Nigella sativa* L. Orient. Pharm. Exp. Med. 19(2):115–129. doi:10.1007/s13596-019-00363-3.

Jabłońska-Trypuć A, Matejczyk M, and SR. 2016. Matrix metalloproteinases (MMPs), the main extracellular matrix (ECM) enzymes in collagen degradation, as a target for anticancer drugs. J. Enzyme Inhib. Med. Chem. 31(sup1):177–183. doi:10.3109/14756366.2016.1161620.

Kang E, Lee DH, Jung YJ, Shin SY, Koh D, Lee YH. 2016. α-Pinene inhibits tumor invasion through downregulation of nuclear factor (NF)-κB-regulated matrix metalloproteinase-9 gene expression in MDA-MB-231 human breast cancer cells. Appl. Biol. Chem. 59(4):511–516. doi:10.1007/s13765-016-0175-6.

Karagiannis GS, Condeelis JS, Oktay MH. 2018. Chemotherapy-induced metastasis: Mechanisms and translational opportunities. Clin. Exp. Metastasis 35(4):269–284. doi:10.1007/s10585-017-9870-x.

Kazemi M. 2014. Phytochemical composition, antioxidant, anti-inflammatory and antimicrobial activity of *Nigella sativa* L. essential oil. J. Essent. Oil-Bearing Plants 17(5):1002–1011. doi:10.1080/0972060X.2014.914857.

Kumari S, Badana AK, Murali Mohan G, Shailender G, Malla RR. 2018. Reactive oxygen species: A key constituent in cancer survival. Biomark. Insights 13:1177271918755391. doi:10.1177/1177271918755391.

Larasati YA, Yoneda-Kato N, Nakamae I, Yokoyama T, Meiyanto E, Kato JY. 2018. Curcumin targets multiple enzymes involved in the ROS metabolic pathway to suppress tumor cell growth. Sci. Rep. 8(1):1–13. doi:10.1038/s41598-018-20179-6.

Liao Z, Chua D, Tan NS. 2019. Reactive oxygen species: A volatile driver of field cancerization and metastasis. Mol. Cancer 18:65. doi:10.1186/s12943-019-0961-y.

Ma J, Peng C. 2024. *Nigella sativa* plant extract inhibits the proliferation of MDA-MB-231 breast cancer cells via apoptosis and cell cycle arrest. Bangladesh J. Pharmacol. 19(1):29–38. doi:10.3329/bjp.v19i1.71069.

Mahmoudvand H, Sepahvand A, Jahanbakhsh S, Ezatpour B, Ayatollahi Mousavi SA. 2014. Evalu-

ation of antifungal activities of the essential oil and various extracts of *Nigella sativa* and its main component, thymoquinone against pathogenic dermatophyte strains. J. Med. Mycol. 24(4):e155–61. doi:10.1016/j.mycmed.2014.06.048.

Marioli-Sapsakou GK, Kourti M. 2021. Targeting production of reactive oxygen species as an anticancer strategy. Anticancer Res. 41(12):5881–5902. doi:10.21873/anticanres.15408.

Mehraj T, Elkanayati RM, Farooq I, Mir TM. 2022. Chapter 4 - A review of *Nigella sativa* and its active principles as anticancer agents. Elsevier. p. 91–118.

Mileo AM, Miccadei S. 2016. Polyphenols as modulator of oxidative stress in cancer disease: New therapeutic strategies. Oxid. Med. Cell. Longev. 2016:6475624. doi:10.1155/2016/6475624.

Mohammed SJ, Amin HH, Aziz SB, Sha AM, Hassan S, Abdul Aziz JM, Rahman HS. 2019. Structural characterization, antimicrobial activity, and *in vitro* cytotoxicity effect of black seed oil. Evidence-based Complement. Altern. Med. 2019:6515671. doi:10.1155/2019/6515671.

Mollazadeh H, Afshari AR, Hosseinzadeh H. 2017. Review on the potential therapeutic roles of *Nigella sativa* in the treatment of patients with cancer: Involvement of apoptosis: - Black cumin and cancer -. J. Pharmacopuncture 20(3):158–172. doi:10.3831/KPI.2017.20.019.

Mori K, Uchida T, Yoshie T, Mizote Y, Ishikawa F, Katsuyama M, Shibanuma M. 2019. A mitochondrial ROS pathway controls matrix metalloproteinase 9 levels and invasive properties in RAS-activated cancer cells. FEBS J. 286(3):459–478. doi:10.1111/febs.14671.

Mukhtar H, Qureshi AS, Anwar F, Mumtaz MW, Marcu M. 2019. *Nigella sativa* L. seed and seed oil: potential sources of high-value components for development of functional foods and nutraceuticals/pharmaceuticals. J. Essent. Oil Res. 31(3):171–183. doi:10.1080/10412905.2018.1562388.

Çınar r, Gıdık B, Dirican E. 2024. Determination of anticancer effects of *Nigella sativa* seed oil on MCF7 breast and AGS gastric cancer cells. Molecular Biology Reports 51(1):491. doi:10.1007/s11033-024-09453-1.

Nurrachma MY, Maran GG, Putri NB, Esti YF, Hermawan A, Meiyanto E, Jenie RI. 2020. Fingerroot (*Boesenbergia pandurata*) extract inhibits proliferation and migration of 4T1 metastatic breast cancer cells. Indones. J. Cancer Chemoprevention 11(3):141–150. doi:10.14499/indonesianjcanchemoprev11iss3pp103-114.

Piskounova E, Agathocleous M, Murphy MM, Hu Z, Huddlestun SE, Zhao Z, Leitch AM, Johnson TM, DeBerardinis RJ, Morrison SJ. 2015. Oxidative stress inhibits distant metastasis by human melanoma cells. Nature 527(7577):186–191. doi:10.1038/nature15726.

Ramadani RD, Utomo RY, Hermawan A, Meiyanto E. 2018. Curcumin analog pentagamaboronon-0-sorbitol inhibits cell migration activity of triple-negative breast cancer cell line. Indones. J. Cancer Chemoprevention 9(3):126–133. doi:10.14499/indonesianjcanchemoprev9iss3pp126-133.

Reczek CR, Chandel NS. 2017. The two faces of reactive oxygen species in cancer. Annu. Rev. Cancer Biol. 1:79–98. doi:10.1146/annurev-cancerbio-041916-065808.

Shokri H. 2016. A review on the inhibitory potential of *Nigella sativa* against pathogenic and toxigenic fungi. Avicenna J. Phytomed. 6(1):21–33.

Toth M, Sohail A, Fridman R. 2012. Assessment of gelatinases (MMP-2 and MMP-9) by gelatin zymography. Methods Mol. Biol. 878:121–135. doi:10.1007/978-1-61779-854-2_8.

Usmani A, Mishra A, Arshad M, Jafri A. 2019. Development and evaluation of doxorubicin self nanoemulsifying drug delivery system with *Nigella sativa* oil against human hepatocellular carcinoma. Artif. Cells, Nanomedicine Biotechnol. 47(1):933–944. doi:10.1080/21691401.2019.1581791.

Vermot A, Petit-Härtlein I, Smith SM, Fieschi F. 2021. NADPH oxidases (Nox): An overview from discovery, molecular mechanisms to physiology and pathology. Antioxidants 10(6):890. doi:10.3390/antiox10060890.

Wisdom KM, Adebowale K, Chang J, Lee JY, Nam S, Desai R, Rossen NS, Rafat M, West RB, Hodgson L, Chaudhuri O. 2018. Matrix mechanical plasticity regulates cancer cell migration through confining microenvironments. Nat. Commun. 9(1):4144. doi:10.1038/s41467-018-06641-z.

Xu Q, Li M, Yang M, Yang J, Xie J, Lu X, Wang F, Chen W. 2018. α-pinene regulates miR-221 and induces G2/M phase cell cycle arrest in human hepatocellular carcinoma cells. Biosci. Rep. 38(6):BSR20180980. doi:10.1042/BSR20180980.

Zeller KS, Riaz A, Sarve H, Li J, Tengholm A, Johansson S. 2013. The role of mechanical force and ROS in integrin-dependent signals. PLoS One 8(5):e64897. doi:10.1371/journal.pone.0064897.

Zhang Y, Fan Y, Huang S, Wang G, Han R, Lei F, Luo A, Jing X, Zhao L, Gu S, Zhao X. 2018. Thymoquinone inhibits the metastasis of renal cell cancer cells by inducing autophagy via AMPK/mTOR signaling pathway. Cancer Sci. 109(12):3865–3873. doi:10.1111/cas.13808.

Zhu H, Sarkar S, Scott L, Danelisen I, Trush M, Jia Z, Li YR. 2016. Doxorubicin redox biology: Redox cycling, topoisomerase inhibition, and oxidative stress. React. Oxyg. Species p. 189–198. doi:10.20455/ros.2016.835.