

Assessment of Cellular Responses to Oxidative Stress using MCF-7 Breast Cancer Cells, Black Seed (*N. Sativa L.*) Extracts and H₂O₂

Ibrahim O. Farah*

Department of Biology, Jackson State University, Jackson, MS 39217, USA

*Correspondence to Dr. Ibrahim O. Farah. Email: ibrahim.o.farah@jsums.edu

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Abstract: Black seed (*N. Sativa L.*) is an oriental spice of the family Ranunculaceae that has long been rationally used as a natural medicine for treatment of many acute as well as chronic conditions including cardiovascular disease and immunological disorders. It has been used in the treatment of diabetes, hypertension, and dermatological conditions. There have been very few studies on the effects of *N. Sativa* as a chemoprevention of chronic diseases as well as in cancer prevention and/or therapy. Oxidative stress is a condition that underlies many acute as well as chronic conditions. The combination and role of oxidative stress and antioxidants *in vivo* is still a matter of conjecture. Our objective for the present study was to expose MCF-7 breast cancer cells *in vitro* (as a chronic disease example) to aqueous and alcohol extracts and in combination with H₂O₂ as an oxidative stressor. Measurement of cell survival under various concentrations and mixtures was conducted using standard cell culture techniques, exposure protocols in 96 well plates and Fluorospectrophotometry. Following cellular growth to 90% confluency, exposure to water (WE) and ethanol (AE) extracts of *N. sativa* and H₂O₂ was performed. Cell survival indices were calculated from percent survival using regression analysis. Results showed that the alcohol extract and its mixtures were able to influence the survival of MCF-7 cells (indices ranged from 357.15- 809.50 µg/ml in descending potency for H₂O₂+AE to the mix of 3). In contrast, H₂O₂ alone reduced effectively the survival of MCF-7 cells and the least effective combinations in descending potency were AE+H₂O₂, WE+H₂O₂, AE+WE, and WE+AE+H₂O₂. Mixtures other than AE+H₂O₂ showed possible interactions and loss of potency. In conclusion, *N. Sativa* alone or in combination with oxidative stress was found to be effective (*in vitro*) in influencing the survival of MCF-7 breast cancer cells, unveiling promising opportunities in the field of cancer chemoprevention and/or treatment.

Keywords: Black seed, Oxidative stress, Nigella sativa, Hydrogen peroxide, MCF-7 breast cancer cells

Introduction

Studies showed that oxidative stress plays a pivotal role in the mediation of many adverse progressive health-related events as well as many disease conditions. Oxygen-derived free radicals induce degenerative processes such as cancer and aging. They are involved in many biological processes such as enzymatic reactions (mitochondrial respiratory chain), detoxifying enzymes of the cytochrome system, phagocytosis, prostaglandin synthesis as well as cytopathological reactions triggered by ionizing, visible and near visible UV-light. Free radical generation is unprogrammed but unavoidable due to our reliance on oxidative processes for life that requires the use of oxygen. Through evolution, O₂ have supported the immortal undifferentiated cell, and then lead to a new type of specialized cell with higher levels of O₂ utilization and higher generation of free radicals

with the consequent implications of being a target for peroxidative attack, degeneration and death [1].

Oxidative stress is a condition induced by oxygen and oxygen-derived free radicals commonly known as reactive oxygen species (ROS). Under normal conditions, adequate levels of antioxidants mainly superoxide dismutase (SOD), catalase, glutathione (GSH) peroxidase and reductase maintain the free radical scavenging potential [2, 3]. Free radicals damage DNA through oxidation of guanine basis via peroxy or alkoxy radicals that result in DNA strand breaks affecting cross-linking. Reactive oxygen species (ROS) damage DNA; however, the role of ROS in breast carcinoma may not be limited to the mutagenic activity that derives carcinoma initiation and progression [4].

New cases of breast cancer were estimated at 215,990 with estimated 40,111 deaths from the disease for the year 2004 [5]. Carcinoma cells *in vitro* and *in vivo* are

frequently under persistent oxidative stress potential and oxygen radical generation. Therefore, the possible impact of oxidative stress on the clinical outcome of breast carcinoma is of practical importance to breast cancer etiology and progression [6].

The micro-environmental hypoxia that arises because of the development of solid tumors also acts to promote tumor growth. Complex interactions between tumor cells and macrophage hypoxia-regulated gene products and their associated pathways form the basis for the hypoxia promotion of tumorigenesis and malignant progression [7, 8]. Hypoxia-induced factor-1 (HIF) that is centrally involved in physiological oxygen homeostasis is also activated in the majority of tumors. HIF-1 affects patterns of gene expression and tumor growth, even though precise effects vary between tumor types. Modulation of HIF activity as a target, if correctly applied, may be therapeutically beneficial in tumor therapy [9].

Since the majority of anticancer agents currently in use are cytotoxic in nature with severe side effects and limited efficacy, chemoprevention was recently suggested as an attractive approach to prevent and cure cancer. In recent years, awareness on the benefits of natural products, food additives, vitamin and mineral supplements, to prevent disease including cancer, was increased. Interestingly, agents reported to protect against the development of cancer are often compounds that have been found to induce specific phase II drug-metabolizing enzymes (DMEs) and that these phase II enzymes that are induced are often those that metabolize compounds that generate ROS [10-12].

N. sativa L. (Black Seed), small annual plant of the order Ranunculaceae, is grown throughout much of Asia and Mediterranean region for its seeds [13-15]. It is used as food spice as well as in traditional medicine for conditions such as asthma, flatulence, polio and abdominal pain as well as in stimulating bone marrow and immune cells [16]. It was also cited for destroying cancer cells [17, 18] as well as raising the number of antibody-producing cells and antiproliferative activity [19].

Black seed supports metabolism, improves digestion, increase body tone, stimulates menstrual period, provide quick energy, increase sperm count and encourages hair growth [20]. Recent studies showed its beneficial effects on the blood and blood homeostasis [21, 22], Respiratory system [23, 24], inflammation and fever [25], liver fibrosis and cirrhosis [26, 27], Hepatotoxicity [28], Gastric secretions and ulcer [29], Antioxidant activity, Viruses and bacteria [30-33], Uterine smooth muscles [34], Phagocytic activity, icosanoid generation and membrane lipid peroxidation of leukocytes [35, 36], Contraception [37], cardiovascular action, Antihistamine activity [38], Cisplatin toxicity [39], and Chemical Carcinogenesis [40].

Studies showed that black seed contains 40% fixed oil, a Saponin and up to 1.4% volatile oil (Melathin, Nigellone, Damascene and Tanin), with Myristic acid (0.5%), Palmitic acid (13.7%), Palmitoleic acid (0.1%) Stearic acid (2.6%), Oleic acid (23.7%), Linoleic acid (Omega 6; 57.9%), Linolenic acid (Omega 3; 0.2%), and Arachidonic acid (1.3%). The protein component [41]

consists of Thiamin, Riboflavin, Pridoxine, Niacin, and Folacin besides other components [42-47].

Studies on the biological effects of Thymoquinone (54% of seed oil) include inhibition of cancer cell growth and induction of apoptosis [48-50], nephropathy [51], hematological, liver fibrosis and cirrhosis, anti-inflammatory, analgesic and antipyretic, respiratory, carcinogenesis [52], hepatotoxicity [53, 54]. The beauty of black seed rests in its ability to restore body function and keep homeostasis [55].

Since there are very limited studies on its effects on breast cancer and the adverse effects of current cancer therapies, we designed this study to expose MCF-7 breast cancer cells to water and ethanol extracts of *N. sativa* and H₂O₂ (as an oxidative stress control). Our intent was to study their cytotoxic/protective effects on these cells aiming at measuring the survival of MCF-7 breast cancer cells under the influence of these chemicals and their combinations by using fluorospectrophometric determinations of cellular respiratory activity as an indirect count for cell survival after a 48-hour exposure. In addition measurement of statistical parameters such as dose response survival curve and death end points for potency comparisons were performed.

Materials and Methods

Chemicals

MCF-7 is a continuous cell line from neoplastic tissue. It is estrogen receptor positive and originated from adult human breast adenocarcinoma. MCF-7 cells were purchased from American Type Culture Collection (ATCC). Fluorescein Diacetate (FDA; Molecular Probes Inc, Eugene, Oregon). RBMI-64 was obtained from Sigma Chemical Company and H₂O₂ was from Fisher Scientific Company.

Tissue Culture

The medium was supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin and penicillin. Tissue culture flasks containing fifteen mls of complete medium were seeded with inoculum and incubated at 37°C in CO₂ incubator. Vials containing the MCF-7 cells (cryosafe preserved) were thawed by gentle agitation for 2 minutes in a water bath at 37°C. Preparation of cell cultures followed standard inoculation and incubation protocols on 75 ml tissue culture flasks (Fisher Scientific). On reaching about 90% confluency, cells were washed with phosphate buffered saline (PBS), trypsinized with 10 ml of 0.25% (w/v) trypsin-0.03% (w/v) EDTA, diluted with fresh medium, counted, and seeded (2.5-5 x 10³ cells/ml) in two sets of 96-well microtiter tissue culture plates for cytotoxicity assay.

Experimental Procedure

N. sativa was obtained from a commercial source in the form of black seed. Extraction followed conventional protocols. The seed was grinded with a clean electric grinder. Ten grams of seed powder were added to clean vials where a 100 mls of de-ionized water was added to

one vial and to the other 100 mls of 95% ethanol were added. Vials were placed at 4°C for overnight. The water extract was separated using a pipette and placed on a clean wide pan and was allowed to dry under the hood. The methanol extract was also separated and allowed to completely dry under a separate hood. From the completely dried products from both extracts, 1 gram was re-suspended in de-ionized water and DMSO for water and alcohol extracts respectively (stock solution of 10,000 µg/ml). After complete suspension of each in its respective vehicle, the extracts were filter sterilized into sterile vials. Chemical plates from sterilized alcohol extract (AE), water extract (WE), hydrogen peroxide (H₂O₂) and their combinations were prepared. Ninety-six well chemical plates were used to make the dilutions: 0, 1.95, 3.9, 7.8, 15.62, 31.25, 62.5, 125, 250, 500 and 1000 µg/ml. Individual components and mixtures were added separately to each of the 96-well tissue culture plates with attached cells. These cells were exposed to a total of 200µl of medium containing respective chemical dilutions and were incubated for 48 hours at 37°C. For the combination of products, aliquots from stock solutions were mixed under the laminar flow hood to keep sterility before serial dilutions were made on the respective chemical plates. The cells were treated and incubated following the protocols mentioned above.

The medium was removed, wells were washed with 200 µl PBS, and 100 µl FDA working solution (10 µg/ml in PBS) was added column-wise to each well and

incubated for 1hr. Surviving cells were able to hydrolyze the added non-fluorescent FDA to the fluorescent fluorescein. The absorbance was measured with Fluoroskan Ascent FL (Thermo Labsystems Inc) at excitation/emission wavelengths of 346/432 nm. Values obtained per concentration were converted to percentage cell viability. Regression analysis of a four replicates and three repeats of each data point was performed on percent cell viability and the resulting equations were used to compute the lethal concentration needed to kill 50% of the cells (LC₅₀). Statistical parameters (Statistical software) and graphic representations of the data were also computed and analyzed.

Results

Results on the cytotoxic effect of the alcohol (AE), water (WE) extracts of *N. sativa L.* and H₂O₂ individually on MCF-7 breast cancer cell line after 48 hours exposure by FDA assay and measured as percent survival are shown in fig.1 a, b, and c. As can be seen both extracts and H₂O₂ showed death curves of varying dose-response relationships. The AE extract showed a very sharp dose-dependent decline of cell survival starting at the low concentration of 1.95 µg/ml. A lower surviving population size in comparison to the other two and an end point death between 250 and 500 µg/ml were observed. Data for WE and H₂O₂ showed 7.8 and 125 µg/ml, a medium and highest surviving population sizes, and >1000 µg/ml cell death end point respectively.

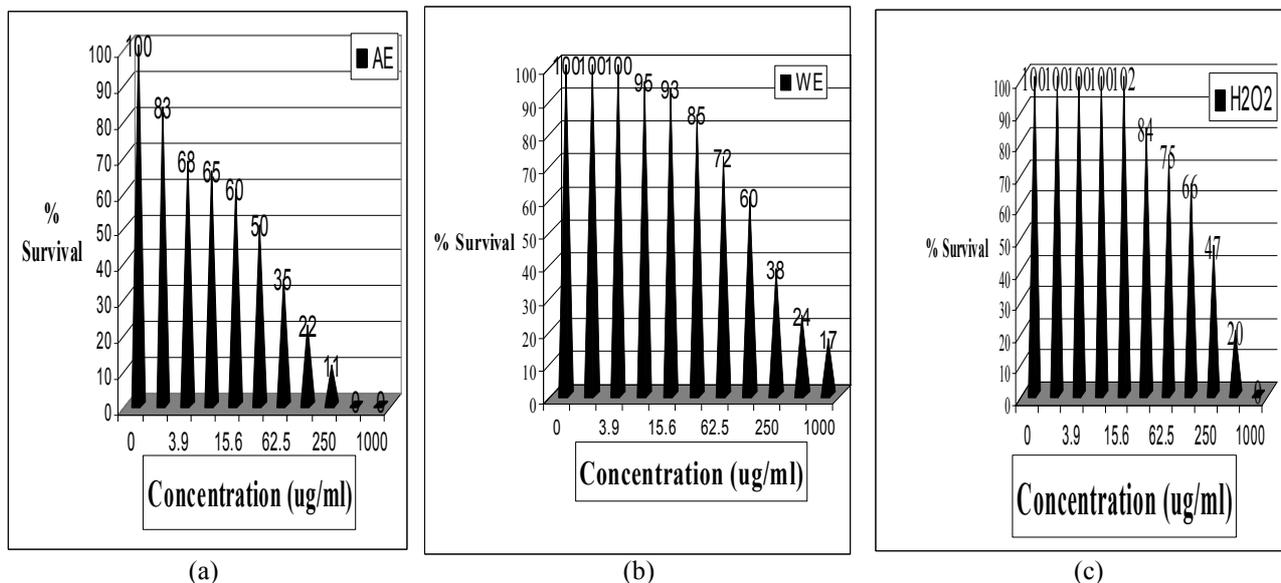


Figure 1: Cellular response of MCF-7 breast cancer cell line to a 48 hour exposure to (a) alcohol extract of black seed (AE) (b) water extract of black seed and (WE) (c) hydrogen peroxide (H₂O₂) in complete RPMI medium and the FDA assay.

Fig 2 shows the results for the combinations of each extract with H₂O₂ and with each other. As can be seen in Fig 2a, the combination of AE and H₂O₂ showed death curves with the strongest dose-response relationship as based on the survival parameters. The AE+H₂O₂ combination showed a very sharp dose-dependent decline of cell survival starting at the low concentration of 1.95 µg/ml. There was a lower surviving population size in comparison to the other two and there was an end point death between 500 and 1000 µg/ml. Data for WE+H₂O₂ and AE+WE showed 7.8 and 62.5 µg/ml, medium and highest surviving population sizes, 500-1000 and >1000 µg/ml cell death endpoint respectively (Fig 2b and c).

Fig 3a shows a comparison of cellular response to the black seed extracts and their respective combinations with H₂O₂. Based on surviving population size; initial effective concentrations; and cell death end points, AE showed the lowest resistant population; the lowest effective concentration; and the lowest cellular death end point. The effectiveness of other products in descending order is AE+H₂O₂, WE+H₂O₂ and WE. Fig 3b shows the pattern of the effectiveness of the mixture of WE+AE (Mix of 2), WE+AE+H₂O₂ (Mix of 3) in comparison to H₂O₂. Both mixtures were inferior to H₂O₂. When comparing the inactivation trends of the two combinations with H₂O₂ as shown in Fig 3c, it is obvious that these trends follow the same pattern of H₂O₂.

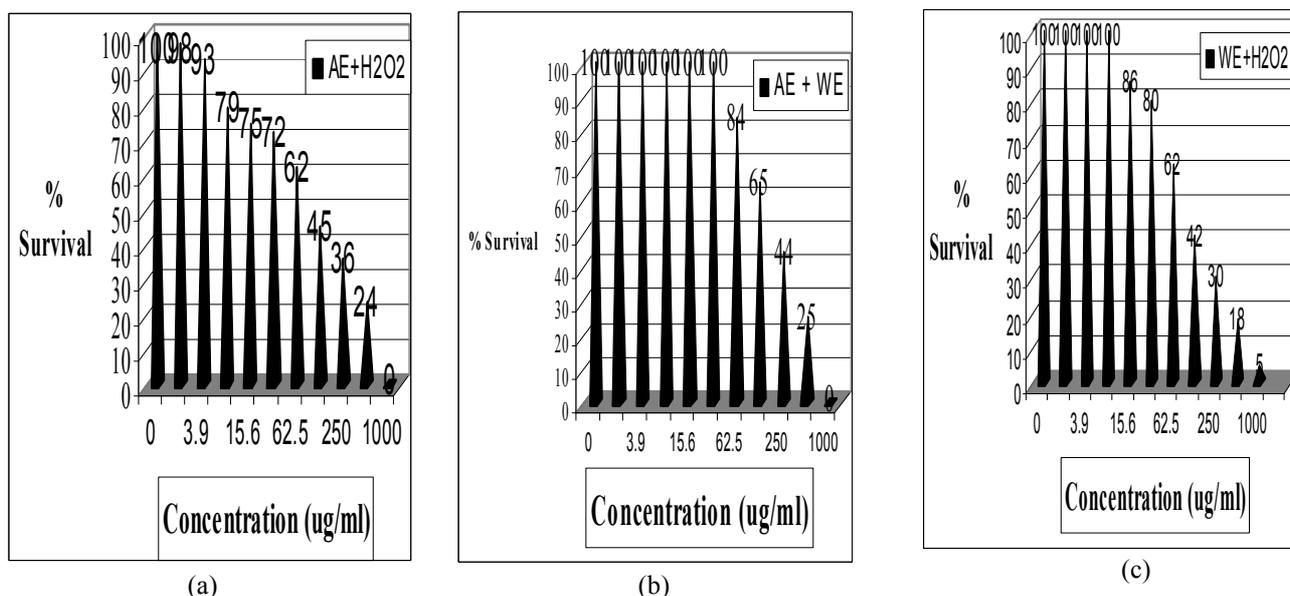


Figure 2: Cellular response of MCF-7 breast cancer cell line to a 48 hour exposure to (a) water extract of black seed and hydrogen peroxide (WE + H₂O₂), (b) alcohol extract of black seed and hydrogen peroxide (AE + H₂O₂) and (c) a mix of alcohol and water extracts of black seed (AE + WE) in complete RPMI medium and the FDA assay.

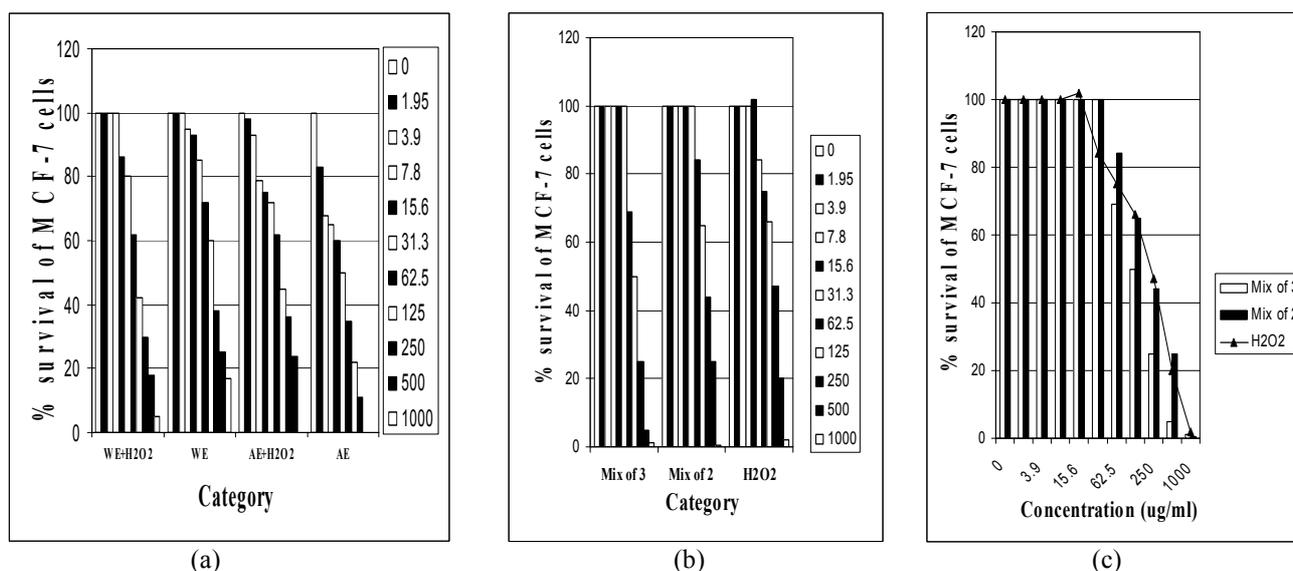


Figure 3: Cellular response of MCF-7 breast cancer cell line to a 48 hour exposure to (a) water, alcohol extract of black seed alone or in combination with H₂O₂, (b) a mixture of water extract, alcohol extract of black seed and H₂O₂, water and alcohol extract of black seed and H₂O₂ and (c) hydrogen peroxide in comparison to the other two mixtures in complete RPMI medium and the FDA assay.

Table 1 shows data on regression equations, R², LC₅₀ means, standard deviation, and 95% confidence interval for the means. As can be seen AE+H₂O₂ showed the lowest LC₅₀ of 357.15, SD of 20.46, 95% CI of 313.98–400.32 µg/ml and R² of 0.94.99 depicting a strong dose-response relation ship. In this regard, AE showed the next stronger response at 418.25, 25.3, 306.42–530.08 µg/ml and 0.9835 respectively. H₂O₂ followed in potency at 636.71, 40.84, 550.54–722.88 µg/ml and 0.9682 respectively. The mixture of H₂O₂ and WE followed at 652.00, 52.28, 541.69–762.31µg/ml and 0.996 respectively. AE+WE at 747.49, 35.02, 676.60–821.38 µg/ml and 0.9811, WE at 796.70, 62.8, 664.19–929.21 µg/ml and 0.9598 and the Mix of 3 at 809.50, 87.49, 624.90–994.10 µg/ml and 0.9692 showed the lowest potency respectively.

Table 1: Linear regression analysis and toxicity index (LC₅₀; µg/ml) of *N. sativa L.* extracts hydrogen peroxide and combinations on MCF-7 breast cancer cells

Treatment	R ²	LC ₅₀	SD	95% CI**
AE+H ₂ O ₂	0.9499	357.15 ^a	20.46	313.98 – 400.32
AE	0.9835	418.25 ^a	25.3	306.42 – 530.08
H ₂ O ₂	0.9682	636.71 ^b	40.84	550.54 – 722.88
WE+H ₂ O ₂	0.9811	652.00 ^b	52.28	541.69 – 762.31
AE + WE	0.996	747.49 ^b	35.02	676.60 – 821.38
WE	0.9598	796.70 ^b	62.8	664.19 – 929.21
Mix of 3	0.9692	809.50 ^b	87.49	624.90 – 994.10

AE= Alcohol Extract. WE= Water Extract.
 H₂O₂= Hydrogen Peroxide.
 ** =Confidence Interval. SD= Standard Deviation.
 ***= AE+WE+H₂O₂. ^a and ^b = Statistical Significance.

Table 2 summarizes the comparative parameters for individual and combinations of compounds as related to their influence on survival and death of MCF-7 breast cancer cells. As can be seen, the maximum resisted concentration (population not affected negatively) ranged between 0 to 62.5 µg/ml and the order of efficacy is AE, AE+H₂O₂, WE, WE+H₂O₂, AE+WE, Mix of 3, and H₂O₂. The first effective concentration (population start of response to chemicals) in order of efficacy is consistent with the first parameter and followed the same sequence. Population size (the dark area of the graph) showed three groups; group 1 is composed of AE and AE+H₂O₂ having the highest efficacy, group 2 is composed of H₂O₂ and WE+H₂O₂ with medium efficacy, and group 3 is composed of WE, AE+WE, and MIX of 3 with the lowest efficacy. The sequence of efficacy for the toxicity index LC₅₀ is AE+H₂O₂, AE, H₂O₂, WE+H₂O₂, AE+WE, WE and Mix of 3.

The efficacy sequence for the slope of the regression line is AE+H₂O₂, AE, WE+H₂O₂, H₂O₂, WE, AE+WE, and Mixes of 3. The Y-intercept will be consistent with the surviving population. Fig 3a shows a comparison of cellular response to the black seed extracts and their respective combination with H₂O₂. Based on surviving population size; initial effective concentrations; and cell death end points, AE showed the lowest resistant population; the lowest effective concentration; and the lowest cellular death end point. The effectiveness of other products in descending order is AE+H₂O₂, WE+H₂O₂ and WE. Fig 3b shows the pattern of the effectiveness of the mixture of WE+AE (Mix of 2), WE+AE+H₂O₂ (Mix of 3) in comparison to H₂O₂. Both mixtures were inferior to H₂O₂. Comparing the inactivation trends of the two combinations with H₂O₂ as shown in Fig 3c, it is obvious that these trends follow the same pattern of H₂O₂ and the sequence of efficacy is AE, WE+H₂O₂, WE, AE+H₂O₂, H₂O₂, Mix of 3 and AE+WE. The death end point of cellular population in terms of efficacy sequence showed AE, AE+H₂O₂, AE+WE, and the same effect for H₂O₂, WE, WE+H₂O₂, and Mix of 3.

Table 2: Comparative parameters of cell survival and cell death of MCF-7 breast cancer cells under the influence of black seed extracts and its combinations with H₂O₂

Parameters	AE	WE	H ₂ O ₂	AE+ H ₂ O ₂	WE+H ₂ O ₂	AE +WE	MIX of 3
Maximum resisted concentration	0	3.9	62.5	0	7.8	31.3	31.3
First effective concentration	1.95	7.8	125	1.95	15.6	62.5	62.5
Population size	1	3	2	1	2	3	3
LC ₅₀	418.25	796.70	636.71	357.15	652.00	747.49	809.50
Slope	-10.51	-18.30	-16.50	-9.53	-15.32	-19.94	-20.49
Y-intercept	107.20	118.30	188.29	119.35	114.00	222.51	215.63
Endpoint concentration	250 - 500	>1000	>1000	500 - 1000	>1000	500 - 1000	>1000

Concentrations are in µg/ml. Mix of 3 = AE+WE+H₂O₂

Discussion

The promising results of recent trials with the antiestrogen tamoxifen and raloxifene showed that not all breast cancers could be prevented or cured with antiestrogens pointing to a need for finding other agents for managing breast cancer. Promising agents, which are currently under study, include retinoids, vitamin D analogues, dihydroepiandrosterone derivatives, NSAIDS and monoterpenes [56-59]. However, from those, only retinoids and NSAIDS were shown to suppress cancer development in animals and humans. Because the majority of anticancer agents currently in use are cytotoxic in nature with severe side effects and limited efficacy, natural products were recently suggested as an attractive approach to prevent and cure cancer. In recent years, awareness on the benefits of natural products, food additives, vitamin and mineral supplements, to prevent disease including cancer, was increased [58-60].

This study aims at assessing and analyzing the cellular responses of MCF-7 breast cancer cells to black seed alcohol and water extracts as well as their combination with each other and H₂O₂. Results on the cytotoxic effects of the alcohol (AE), water (WE) extracts of *N. sativa L.* and H₂O₂ individually on MCF-7 breast cancer cell line after 48 hours exposure by FDA assay and measured as % survival are respectively shown in Fig. 1 a, b, and c.

As can be seen both extracts and H₂O₂ showed death curves depicting varying dose-response relationships. The AE extract showed a very sharp dose-dependent decline of cell survival starting at the low concentration of 1.95 µg/ml, LC₅₀ of 418.25 µg/ml. There was a lower population size in comparison to the other two, and an end point death between 250 and 500 µg/ml. Data for WE and H₂O₂ showed 7.8 and 125 µg/ml, 796.70 and 636.71 µg/ml for LC₅₀'s. There was a medium and highest population sizes, and >1000 µg/ml death end point for both respectively (Tables 1 and 2).

The above data sets consistently confirm that the alcohol extract or the oil portion of black seed was highly efficacious on this cell model and its potency exceeds by far the effects of the other two compounds including H₂O₂. Studies with whole extracts, oil and fractions of black seed (*N. sativa L.*), showed cytotoxic effects to a number of cancer cells [48-50] including MCF-7 breast cancer cells even though the portion used for exposure was Thymoquinone and the assay was the MTT assay. Interestingly and essentially they also found that non-cancerous cells were relatively resistant to thymoquinone; the major component of black seed oil [32].

When we used the combinations of each extract with H₂O₂ and each other as shown in Fig (2a, b and c), AE and H₂O₂ showed death curves; depicting the strongest dose-response relationship based on the survival index (LC₅₀). However, this combination also showed a very sharp dose-dependent decline of cell survival starting at the lowest concentration of 1.95 µg/ml, LC₅₀ of 357.15 µg/ml. There was a lower population size in comparison to the other two, and point death between 500 and 1000

µg/ml. Data for WE+H₂O₂ and AE+WE showed 7.8 and 62.5 µg/ml, 652.00 and 747.40 µg/ml for LC₅₀'s, a medium and highest population sizes, 500-1000 and >1000 µg/ml death point for both respectively (Tables 1 and 2). Comparing the above data with the efficacy of individual product, showed that, even though the LC₅₀ of the AE+H₂O₂ combination was lower than that of AE and H₂O₂ individually, this will show that there is a synergistic effect of the combination. However, adding other parameters to this finding such as population size and end point death, AE out performed AE+ H₂O₂ pointing to that AE may have a killing mechanism other than through the generation of H₂O₂ as a pro-oxidation outcome from its antioxidant constituents. It has been known in the literature that antioxidants may act as pro-oxidants when used at high levels [1, 6].

In this case, there may be production of singlet O₂, hydroxyl radicals and aldehydes, carbonyls or other glycation products from their interaction with cell constituents [10-12]. The WE+ H₂O₂, even though with a higher LC₅₀ and population size, it was found to be as effective as the AE+H₂O₂ concerning end point death findings. However, this combination showed a synergistic effect for only the end point death parameter. On the contrary, the AE+WE combination was found to be inferior in efficacy to AE and AE+H₂O₂ and was superior to WE, H₂O₂, WE+H₂O₂ and the Mix of 3. However, the mix of 3 showed antagonistic effects, which may be due to the interaction within the product before cell exposure, which led to its poor performance and lowest efficacy.

Data from Fig 3a showed a comparison of cellular responses to the black seed extracts and their respective combination with H₂O₂. Based on surviving population size; initial effective concentrations; and cell death end points, AE showed the lowest resistant population; the lowest effective concentration; and the lowest cellular death end point. The effectiveness of other products in descending order is AE+H₂O₂, WE+H₂O₂ and WE. Fig 3b shows the pattern of the effectiveness of the mixture of WE+AE (Mix of 2), WE+AE+H₂O₂ (Mix of 3) in comparison to H₂O₂. Both mixtures were inferior to H₂O₂. Comparing the inactivation trends of the two combinations with H₂O₂ as shown in Fig 3c, it is obvious that these trends follow the same pattern of H₂O₂ suggesting some mechanistic resemblance.

The physiologic role of H₂O₂ includes regulation of cytosolic Ca⁺⁺ levels. It acts as a second messenger for insulin and is powerful inhibitor of glucagon action as well as being an activator of gluconeogenesis. Peroxide induced cell injury induces cell lyses, and the intracellular generation of H₂O₂ may also be the cause of some cytotoxicity of substituted benzoquinones, naphthoquinones, anthacycline quinone antibiotics, nitroaromatic compounds and bipyridyliums form highly autooxidizable radicals following a reductase-catalysed reduction. AE is mainly Thymoquinone, which may explain the synergistic effect of the AE+H₂O₂ combination [1].

The ranking of efficacy as based on all parameters was that AE+H₂O₂ showed the lowest LC₅₀ of 357.15,

SD of 20.46, 95% CI of 313.98–400.32 $\mu\text{g/ml}$ and R^2 of 0.9499 depicting a strong dose response relationship. AE showed the next stronger response at 418.25, 25.3, 306.42–530.08 $\mu\text{g/ml}$ and 0.9835 respectively. H_2O_2 followed in potency at 636.71, 40.84, 550.54–722.88 $\mu\text{g/ml}$ and 0.9682 respectively. The mixture of H_2O_2 and WE followed at 652.00, 52.28, 541.69–762.31 $\mu\text{g/ml}$ and 0.996. AE+WE at 747.49, 35.02, 676.60–821.38 $\mu\text{g/ml}$ and 0.9811, WE at 796.70, 62.8, 664.19–929.21 $\mu\text{g/ml}$ and 0.9598 and the Mix of 3 at 809.50, 87.49, 624.90–994.10 $\mu\text{g/ml}$ and 0.9692 showed the lowest potency respectively (Table 1). Data from table 2 analyze the comparative parameters of all individual and combination compounds as related influence on cell survival and death of MCF-7 breast cancer cells in response to black seed extracts and H_2O_2 .

As can be seen, the maximum resisted concentration (population not affected negatively) ranged between 0 to 62.5 and the order of efficacy in this case is AE, AE+ H_2O_2 , WE, WE+ H_2O_2 , AE+WE, Mix of 3, and H_2O_2 . The first effective concentration (population start of response to chemicals) in order of efficacy is consistent with the first parameter and followed the same sequence. Population size (the dark area of the graph) showed three groups; group 1 is composed of AE and AE+ H_2O_2 having the highest efficacy, group 2 is composed of H_2O_2 and WE+ H_2O_2 with medium efficacy, and group 3 is composed of WE, AE+WE, and mix of 3 with the lowest efficacy. The sequence of efficacy for the toxicity index LC_{50} is AE+ H_2O_2 , AE, H_2O_2 , WE+ H_2O_2 , AE+WE, WE and Mix of 3. The efficacy sequence for the slope of the regression line is AE+ H_2O_2 , AE, WE+ H_2O_2 , H_2O_2 , WE, AE+WE, and Mixes of 3. The y-intercept will be consistent with the surviving population and the sequence of efficacy is AE, WE+ H_2O_2 , WE, AE+ H_2O_2 , H_2O_2 , Mix of 3 and AE+WE. The death end point of cellular population in terms of efficacy sequence showed AE, AE+ H_2O_2 , AE+WE, and the same effect for H_2O_2 , WE, WE+ H_2O_2 , and Mix of 3.

Data displayed above showed that *N. sativa* extracts did not protect MCF-7 cell line against H_2O_2 . On the contrary, both extracts and their combinations showed a potential for killing these cells in a dose-dependent fashion. Even though differences were not statistical within LC_{50} s for H_2O_2 , WE+ H_2O_2 , WE, AE+WE, and Mix of 3, there was a significant difference between this group and the group of AE, AE+ H_2O_2 (Table 1). Even though there was no significant statistical difference between AE and AE+ H_2O_2 there was an indication as based on other parameters such as slope, y-intercept and death end points that the combination of AE and H_2O_2 did enhance their efficacy (LC_{50} of 357.15 for both vs. 418.25 and 636.71 $\mu\text{g/ml}$ for AE and H_2O_2 respectively (Table 2). Statistical analysis of data showed significant ($p < 0.05$) statistical differences between H_2O_2 , AE and their combinations in comparison to those who have water based vehicle. This could be attributed to the presence of either a protective chain breaking antioxidant in the water extract or the antagonistic interference of

these chemicals with the resultant loss of potency in the mixture [61, 62, and 63].

This study also shows that studies using LC_{50} , as death indicators are not relevant to the total eradication of cancer cells. These cells have the potential to re-grow and if not coupled with end point studies, there is no way of ascertaining the effectiveness of medications or chemotherapeutic agents used for cancer treatment. Even though we did not involve normal or non-cancerous cells in this study, it is worth mentioning that studies with these types of cells are essential to elucidate the usefulness of this product for cancer prevention and/or treatment.

From this study and studies by others [8-12, 17-19, 48], it is apparent that the most potent part of *N. sativa* for cytotoxicity to cancer cells is the alcohol extracted oil portion of the seed. Future studies should focus on this portion and mechanisms of cellular death established with cancerous, non-cancerous and normal cells to confirm the selective and/or differential cytotoxicity of the product.

Conclusions

N. sativa alone or in combination with oxidative stress was effective in inactivating breast cancer cells, unveiling opportunities for promising results in the field of prevention and treatment of cancer. Data presented here showed that the most potent part of *N. sativa* for cytotoxicity to cancer cells is the alcohol extracted oil portion of the seed. Future studies should focus on this portion and mechanisms of cellular death established with cancerous, non-cancerous and normal cells to confirm the selective and/or differential cytotoxicity of the product. The data also showed the limitations of using LC_{50} as indices for effective cellular death in cancer. One of the interesting findings of this study that extends its applicability is that *N. sativa* active portion (being a natural product), can utilize the internal oxidative stress state of cancer cells to increase its killing potency. Further Studies are needed to focus on elucidation of cell death mechanisms due to *N. sativa* and the characterization of its role as a candidate for cancer prevention and/or treatment.

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