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Research Article

Synthesis, Characterisation, Invitro anticancer activity of Diazo Derivatives of 1,3,4-oxadiazole

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Abstract

This study delves into the comprehensive evaluation of newly synthesized Diazo Derivatives of 1,3,4-oxadiazole compounds, including Aniline, Ortho nitro aniline, Meta nitro aniline, Ortho anisidine, Meta anisidine, 3-chloro aniline, 2,6-dimethyl aniline, 2,5-dichloro aniline, and 3,5-dichloro aniline, aiming to unravel their potential as antioxidants and anticancer agents. The assessment of antioxidant potential involved the utilization of the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. The cytotoxic activity of these compounds was assessed against MCF-7 breast cancer cells using the MTT assay. Morphological assessments using acridine orange/ethidium bromide (AO/EB) staining. Results were expressed as IC₅₀ values, with 2,5-dichloro aniline and 3-chloro aniline demonstrating remarkable antioxidant activity, each with an IC₅₀ value of 62.5 µg/mL. Several other compounds also exhibited significant antioxidant properties, albeit with slightly higher IC₅₀ values. Notably, Ortho anisidine and Ortho nitro aniline displayed no substantial inhibitory activity against oxidative stress. The cytotoxic activity of these compounds was assessed against MCF-7 breast cancer cells using the MTT assay, with IC₅₀ values ranging from 62.5 to 1000 µg/mL. 2,5-dichloro aniline and 3-chloro aniline emerged as potent inhibitors, both achieving an IC₅₀ value of 62.5 µg/mL. Aniline, Meta nitro aniline, Meta anisidine, 2,6-dimethyl aniline, and 3,5-dichloro aniline exhibited intermediate inhibitory activity, while Ortho anisidine and Ortho nitro aniline showed no significant inhibition. Morphological assessments using acridine orange/ethidium bromide (AO/EB) staining revealed diverse cellular responses induced by 2,5-dichloro aniline and 3-chloro aniline treatment. These changes align with well-established apoptotic processes observed in therapeutically treated cell lines. This research contributes valuable insights into the antioxidant and anticancer potential of Diazo Derivatives of 1,3,4-oxadiazole compounds, with 2,5-dichloro aniline and 3-chloro aniline emerging as promising candidates for further exploration in combatting oxidative stress and breast cancer. These findings underscore the importance of antioxidants in cancer prevention and the potential therapeutic applications of these compounds.

Keywords: Diazo Derivatives of 1,3,4-oxadiazole, 2,5-dichloro aniline, 3-chloro aniline, DPPH, MTT assay, MCF-7

INTRODUCTION

Cancer is a complex and devastating group of diseases characterized by the uncontrolled growth and spread of abnormal cells within the body¹. One of the foremost difficulties in treating cancer is its heterogeneity. Cancer is not a singular disease but a collection of diseases, each with distinct characteristics, molecular profiles, and behaviours². This diversity is seen in different cancer types, such as breast, lung, prostate, and colon cancer, each requiring a tailored approach to treatment. Cancer cells are notorious for their ability to adapt, evolve, and evade the body's immune system and conventional therapies³. They can develop mechanisms to resist chemotherapy, radiation therapy, and targeted therapies, making it difficult to eradicate them completely. This adaptability results in treatment resistance and disease recurrence, which are common and frustrating aspects of cancer management⁴. Cancer is a multifaceted disease with numerous difficulties that hinder its effective treatment. Its heterogeneity, adaptability, late-stage diagnosis, physical and

psychological burdens, financial costs, and intricate biology all contribute to the complex landscape of cancer treatment⁵. Despite these challenges, on-going research, advances in drug discovery, and a multidisciplinary approach to cancer care offer hope for improved outcomes and the development of more effective therapies in the future.

Diazo derivatives of 1,3,4-oxadiazole compounds have garnered significant attention in the field of cancer research due to their potential as anticancer agents⁶. These compounds belong to a class of organic molecules characterized by the presence of an oxadiazole ring containing a diazo group (-N=N-). While research in this area is ongoing, these derivatives have shown promising results in preclinical studies, and they offer a novel approach to cancer treatment⁷. Diazo derivatives of 1,3,4-oxadiazole compounds have been found to exhibit anticancer activity through various mechanisms. They may interfere with the DNA replication process, leading to DNA damage and apoptosis (programmed cell death) in cancer cells. Some derivatives have shown the ability to inhibit specific enzymes or signalling pathways that are crucial for

cancer cell survival and proliferation. One of the advantages of these compounds is their potential for selective cytotoxicity, meaning they can specifically target cancer cells while sparing healthy cells. This selectivity is essential to minimize side effects associated with cancer treatments. Diazo derivatives of 1,3,4-oxadiazole compounds can be used in combination with existing cancer therapies, such as chemotherapy or radiation therapy. This may enhance treatment efficacy and reduce the likelihood of drug resistance⁸. The structural diversity of diazo derivatives allows for the development of a wide range of compounds with varying properties. Researchers can fine-tune the chemical structure to optimize drug-like properties and increase their effectiveness against specific cancer types. Diazo derivatives of 1,3,4-oxadiazole compounds represent a promising avenue in cancer research and drug development⁹. Their unique chemical structure and potential for selective cytotoxicity make them attractive candidates for further investigation.

MATERIAL AND METHODS

Aniline, Ortho nitro aniline, Meta nitro aniline, Ortho anisidine, Meta anisidine, 3-chloro aniline, 2,6-dimethyl aniline, 2,5-dichloro aniline and 3,5- dichloro aniline, Acridine orange, Hexamethyldisilane (HMDS), Sterile cotton swabs, Ethanol, DPPH, DMEM (Dulbecco's Modified Eagle's medium (DMEM),

$$\text{Diazo derivatives of 1,3,4 – oxadiazole (DPPH)} = \frac{A_c \text{ control} - A_s \text{ of antioxidant}}{A_c \text{ control}} \times 100$$

Anticancer activity of Diazo Derivatives of 1,3,4-oxadiazole compounds -MTT assay

To enumerate active MCF-7 breast cancer cells, a hemocytometer was employed in this study. These active cells were subsequently seeded into sterile 96-well plates at a concentration of 1×10^4 cells/ml in each well and allowed to incubate for a duration of 24 hours to facilitate their adherence and growth. Subsequently, the breast cancer cells were subjected to treatment with varying concentrations of

$$\text{Half – maximal inhibitory concentration (IC50)} = \frac{A_c \text{ control} - A_s \text{ of treated}}{A_c \text{ control}} \times 100$$

2,5-dichloro aniline and 3-chloro aniline apoptotic induction was measured by acridine orange/ethidium bromide (AO/EB) staining.

Breast cancer cells were initially seeded in sterile six-well plates at a concentration of 5×10^4 cells per well and then incubated for a period of 24 hours. Following this incubation, the cells underwent a thorough washing with PBS for 24 hours, effectively eliminating any deceased or undetected cells from the culture. Subsequently, these cells were subjected to treatment with varying concentrations of 2,5-dichloro aniline and 3-chloro aniline, ranging from 125 µg/ml down to 7.785 µg/ml. After an additional 24-hour incubation period, the cells were gently detached and subjected to further washing with sterile PBS. Any cells that did not detach were then exposed to a staining solution composed of AO/EB (100 µg/ml) in a 1:1 ratio for a duration of 5 minutes at room temperature. Subsequently, these AO/EB-stained cells were meticulously examined under a fluorescence microscope at a 45x magnification to facilitate the identification of live and dead cells. The number of cells exhibiting characteristics indicative of apoptosis was quantified in relation to the total cell population observed within the microscope's field of view. This experimental approach allowed for the assessment of the impact of varying concentrations of 2,5-dichloro aniline and 3-

antibiotics (streptomycin and penicillin-G), amino acids (L-glutamine), phosphate-buffered saline (PBS), DPPH (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide, 2',7'-diacetyl dichloro fluorescein), SDS (sodium dodecyl sulfate), trypan blue, trypsin-EDTA, acridine orange, ethidium bromide, rhodamine-123, Triton X-100, dimethyl sulfoxide (DMSO), and bovine serum albumin.

Antioxidant activity

In a study conducted by Muddukrishnaiah et al. in 2015, the DPPH radical scavenging activity was assessed with slight adjustments¹⁰. The experimental procedure involved the mixing of 100 µL of various dilutions of compounds, namely Aniline, Ortho nitro aniline, Meta nitro aniline, Ortho anisidine, Meta anisidine, 3-chloro aniline, 2,6-dimethyl aniline, 2,5-dichloro aniline, and 3,5- dichloro aniline, with 0.1 ml of a 0.16 mM solution of DPPH. To evaluate antioxidant activity, 96-well plates were placed in a dark environment for a duration of 30 minutes. Following this incubation period, the absorbance of the mixture was measured at a wavelength of 517 nm using an automated microplate reader. A_c is the absorbance of the control – DPPH. Absorbance of the Aniline, Ortho nitro aniline, Meta nitro aniline, Ortho anisidine, Meta anisidine, 3-chloro aniline, 2,6-dimethyl aniline, 2,5- dichloro aniline and 3,5-dichloro aniline.

Aniline, Ortho nitro aniline, Meta nitro aniline, Ortho anisidine, Meta anisidine, 3-chloro aniline, 2,6-dimethyl aniline, 2,5-dichloro aniline, and 3,5- dichloro aniline, spanning from 50 to 2.5 µl/ml. Following this treatment, the plates were incubated at a temperature of 37°C for 24 hours under conditions of 95% humidity in sterile air, supplemented with 5% CO₂. Subsequent to this incubation period, the cells were subjected to treatment with the MTT reagent¹¹. The percentage of cell viability was determined using the subsequent formula:

chloro aniline on breast cancer cells, with particular attention paid to the induction of apoptosis-related changes in the cell population.

RESULTS AND DISCUSSION

Antioxidant activity (DPPH) of Diazo Derivatives of 1,3,4-oxadiazole compounds

In this research endeavour, a set of newly synthesized Diazo Derivatives of 1,3,4-oxadiazole compounds (Aniline, Ortho nitro aniline, Meta nitro aniline, Ortho anisidine, Meta anisidine, 3-chloro aniline, 2,6-dimethyl aniline, 2,5- dichloro aniline and 3,5- dichloro aniline) Figure 1 underwent a comprehensive assessment to gauge their in vitro antioxidant capabilities, with the goal of quantifying their potential as antioxidants. The evaluation hinged on the deployment of the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay; a widely-used method employed to gauge the capacity of compounds to counteract oxidative processes. This assay precisely measures the reduction of DPPH, a stable free radical, in response to the introduction of these synthesized compounds. The results were expressed in terms of the concentration (in µg/mL) of each compound required to achieve a 50% reduction in the DPPH color change, a pivotal parameter that reflects their antioxidant potency^{12,13}.

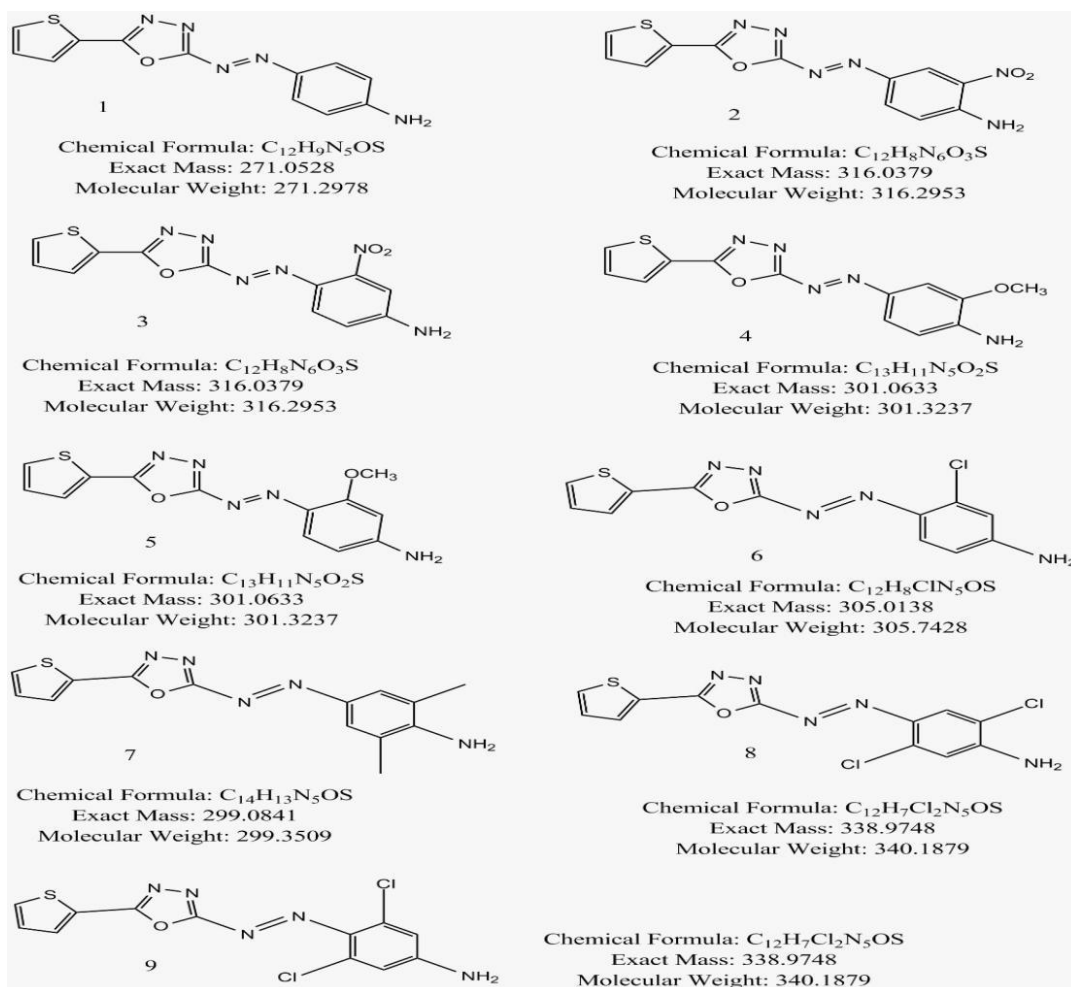


Figure 1: Diazo Derivatives of 1,3,4-oxadiazole compounds (Aniline, Ortho nitro aniline, Meta nitro aniline, Ortho anisidine, Meta anisidine, 3-chloro aniline, 2,6-dimethyl aniline, 2,5- dichloro aniline and 3,5- dichloro aniline)

Additionally, Aniline, Meta nitro aniline, Meta anisidine, 2,6-dimethyl aniline, and 3,5-dichloro aniline demonstrated distinct levels of activity each having IC_{50} values of 31.25 $\mu\text{g}/\text{ml}$ to 62.5 $\mu\text{g}/\text{mL}$. 2,5-dichloro aniline and 3-chloro aniline showing good antioxidant activity compared to the ascorbic acid (IC_{50} values of 15.56 $\mu\text{g}/\text{ml}$).

On the other hand, Ortho anisidine and Ortho nitro aniline did not exhibit any significant inhibitory activity against MCF-7 cells. In other words, these two compounds were ineffective in inhibiting the target at the concentrations tested (Figure 2).

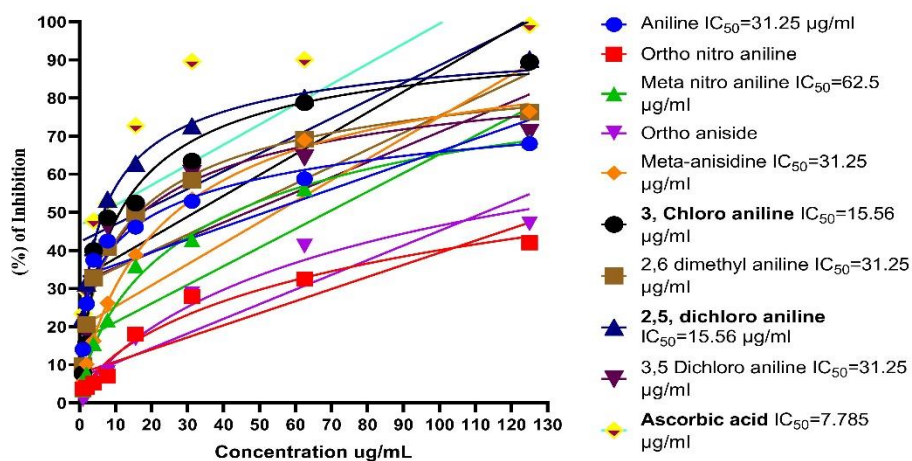


Figure 2: IC_{50} of the antioxidant activity of the synthesised compounds and compared with ascorbic acid. The data is expressed as the mean value along with the standard deviation, which was computed from three independent experiments. When compared to the control group, * $P < 0.05$ indicates statistical significance.

Antioxidants are naturally occurring compounds that play a crucial role in protecting our cells and tissues from oxidative damage ¹⁴. They work by neutralizing harmful molecules known as free radicals, which have the potential to cause cellular damage and contribute to various diseases, including cancer. Oxidative stress is a condition characterized by an excess of free radicals in the body, overwhelming the body's natural defence mechanisms. Prolonged oxidative stress can lead to DNA mutations, protein damage, and lipid peroxidation, all of which are associated with the development of cancer.

Antioxidants help safeguard the integrity of our DNA, preventing mutations that can lead to the uncontrolled cell growth characteristic of cancer ¹⁵. By reducing DNA damage, antioxidants contribute to maintaining the genetic stability of cells. Some antioxidants have been shown to inhibit the initiation of tumors ¹⁶. For example, certain phytochemicals found in fruits and vegetables, such as polyphenols and carotenoids, possess antioxidant properties and may reduce the risk of cancer by preventing the early stages of tumor formation. Chronic inflammation is a known contributor to cancer development, and antioxidants can help mitigate inflammation by neutralizing free radicals that fuel inflammatory processes ¹⁷. This anti-inflammatory effect can be instrumental in cancer prevention. Antioxidants can

support the immune system's ability to identify and eliminate cancer cells. By reducing oxidative stress, antioxidants may help maintain an optimal immune response against malignant cells.

Antioxidant activity is integral to cancer prevention because it helps counteract the damaging effects of oxidative stress, inflammation, and DNA damage -key factors in cancer development. Incorporating a balanced diet rich in antioxidants and adopting a healthy lifestyle can contribute significantly to reducing the risk of cancer and promoting overall well-being.

MTT assay of Newly synthesised Diazo Derivatives of 1,3,4-oxadiazole derivatives

just created Diazo Using the MTT method, derivatives of 1,3,4-oxadiazole were evaluated in vitro for their inhibitory effect against anticancer cell lines including MCF-7 (human breast cancer). It was expressed as ($\mu\text{g}/\text{mL}$) and shown in Figure 1 how much of a drug was required to stop 50% of cancer cells from growing. On evaluated human tumour cells, the nine examined substances had varying degrees of inhibitory effects. The ability of various substances to inhibit MCF-7 cells was tested, including aniline, ortho nitro aniline, meta nitro aniline, ortho anisidine, meta anisidine, 3-chloro aniline, 2,6-dimethyl aniline, 2,5-dichloro aniline, and 3,5-dichloro aniline (Figure 3).

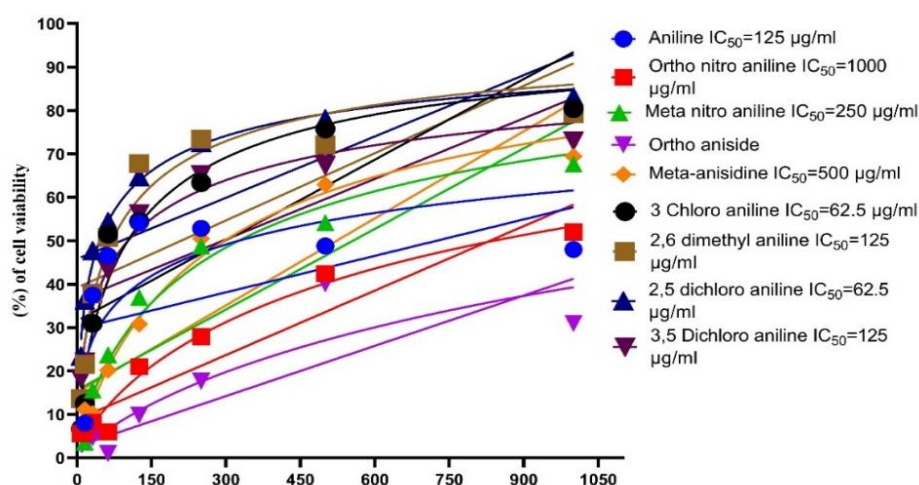


Figure 3: IC₅₀ of the cytotoxic activity of the examined compounds against human breast cancer (MCF-7 cells). The data is expressed as the mean value along with the standard deviation, which was computed from three independent experiments. When compared to the control group, *P<0.05 indicates statistical significance.

The synthesized compounds were evaluated for their inhibitory potency, and the results fell within a range of 62.5 to 1000 $\mu\text{g}/\text{mL}$. Among these compounds, 2,5-dichloro aniline and 3-chloro aniline displayed particularly noteworthy inhibitory activity, both achieving an IC₅₀ value of 62.5 $\mu\text{g}/\text{mL}$. This means that these two compounds were highly effective in inhibiting the target, with a lower concentration required for half-maximal inhibition compared to the other compounds.

Additionally, Aniline, Meta nitro aniline, Meta anisidine, 2,6-dimethyl aniline, and 3,5-dichloro aniline demonstrated distinct levels of activity, each having IC₅₀ values of 250 $\mu\text{g}/\text{mL}$ or 125 $\mu\text{g}/\text{mL}$. These values indicate that these compounds were also effective inhibitors, though they required a somewhat higher concentration to achieve half-maximal inhibition compared to 2,5-dichloro aniline and 3-chloro aniline. On the other hand, Ortho anisidine and Ortho nitro aniline did not exhibit any significant inhibitory activity against MCF-7 cells. In other words, these two compounds

were ineffective in inhibiting the target at the concentrations tested.

The synthesized compounds displayed a range of inhibitory activities, with 2,5-dichloro aniline and 3-chloro aniline standing out as the most potent inhibitors, followed by Aniline, Meta nitro aniline, Meta anisidine, 2,6-dimethyl aniline, and 3,5-dichloro aniline with intermediate levels of activity. In contrast, Ortho anisidine and Ortho nitro aniline did not show any inhibitory activity against the tested cells. These findings provide valuable insights into the potential use of these compounds for inhibiting the target in MCF-7 cells, with the most promising candidates being 2,5-dichloro aniline and 3-chloro aniline with IC₅₀ value 62.5 $\mu\text{g}/\text{mL}$. As reported by Buranrat, Benjaporn et al. ¹⁸, MCF-7 cells underwent doxorubicin treatment, and their viability was assessed through the MTT assay. The results unveiled a significant reduction in cell growth within a 24-hour treatment window,

with an observed IC_{50} value of $5.2 \pm 0.2 \mu\text{g/mL}$, demonstrating statistical significance at $P < 0.05$.

AO/EB 2,5-dichloro aniline and 3-chloro aniline

In order to investigate potential morphological alterations in MCF-7 cells following treatment with 2,5-dichloro aniline and 3-chloro aniline, a fluorescence staining technique utilizing acridine orange/ethidium bromide (AO/EB) was employed. This staining method, as depicted in Figure 4, was used to examine, and assess the apoptotic characteristics induced by these compounds in MCF-7 cells. The AO/EB staining technique provided valuable insights into cell viability and

membrane integrity. It relies on the differential permeability of fluorescent dyes into cells. Live cells typically allow AO to permeate, resulting in a green fluorescence, whereas dead cells tend to be more permeable to EB, leading to an orange-red fluorescence. In the context of this study, the AO/EB staining fluorescence patterns revealed distinctive cellular states: A) Viable cells, characterized by well-organized nuclei, emitted a green fluorescence. B and C early apoptotic cells, showing nuclear condensation, exhibited an orange-green fluorescence and late apoptotic cells, with highly condensed or fragmented chromatin, fluoresced in shades ranging from orange to red (Figure 4).

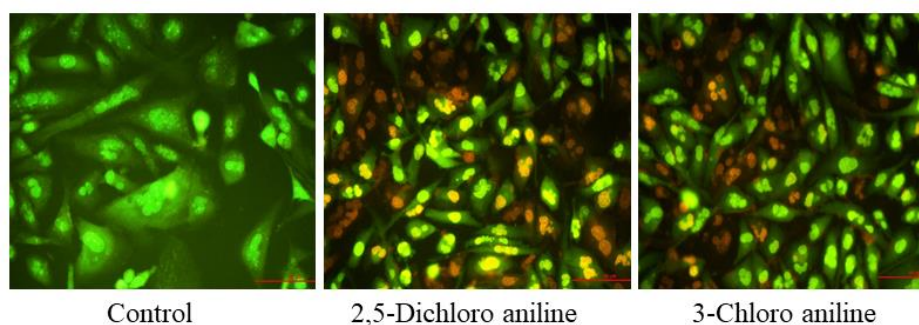


Figure 4: A: MCF-7 control cells; B: 2,5-dichloro aniline treated MCF-7 cells; C: 3-chloro aniline treated MCF-7 cells.

The observed variations in cytological and morphological changes within the nuclei, as revealed by AO/EB staining, provided clear indications of the diverse cellular responses triggered by the treatment with 2,5-dichloro aniline and 3-chloro aniline. These changes in staining patterns align with the well-established and documented apoptotic processes that are frequently observed in cell lines undergoing therapeutic treatments.

CONCLUSION

This research assessed the in vitro antioxidant and cytotoxic activities of newly synthesized Diazo Derivatives of 1,3,4-oxadiazole compounds. The compounds, including Aniline, Ortho nitro aniline, Meta nitro aniline, Ortho anisidine, Meta anisidine, 3-chloro aniline, 2,6-dimethyl aniline, 2,5-dichloro aniline, and 3,5-dichloro aniline, were evaluated using the DPPH assay. 2,5-dichloro aniline and 3-chloro aniline showed remarkable antioxidant activity, with IC_{50} values of $62.5 \mu\text{g/mL}$. Aniline, Meta nitro aniline, Meta anisidine, 2,6-dimethyl aniline, and 3,5-dichloro aniline showed significant antioxidant activity with slightly higher IC_{50} values. Ortho anisidine and Ortho nitro aniline did not exhibit significant inhibitory activity against oxidative stress. The MTT assay assessed the cytotoxic activity of these compounds against MCF-7 breast cancer cells, with 2,5-dichloro aniline and 3-chloro aniline being the most potent inhibitors. Aniline, Meta nitro aniline, Meta anisidine, 2,6-dimethyl aniline, and 3,5-dichloro aniline exhibited intermediate levels of activity, with IC_{50} values ranging from $125 \mu\text{g/mL}$ to $250 \mu\text{g/mL}$. Ortho anisidine and Ortho nitro aniline displayed no significant inhibitory activity against MCF-7 cells. AO/EB staining revealed distinct cellular states of MCF-7 cells induced by 2,5-dichloro aniline and 3-chloro aniline, including viable cells with organized nuclei, early apoptotic cells with nuclear condensation, and late apoptotic cells with highly condensed or fragmented chromatin. These observations support the diverse cellular responses induced by these compounds, consistent with well-documented apoptotic processes in therapeutically treated cell lines.

The synthesized Diazo Derivatives of 1,3,4-oxadiazole compounds exhibited varying degrees of antioxidant and cytotoxic activities, with 2,5-dichloro aniline and 3-chloro aniline demonstrating notable potential as antioxidants and inhibitors of MCF-7 breast cancer cells. These findings contribute valuable insights into the potential utility of these compounds for their roles in combating oxidative stress and cancer-related conditions, warranting further exploration for potential therapeutic applications. Further In-vivo studies required for the study of toxicity and efficacy of the synthesised compounds for therapeutic applications.

Conflict of Interest: Nil

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