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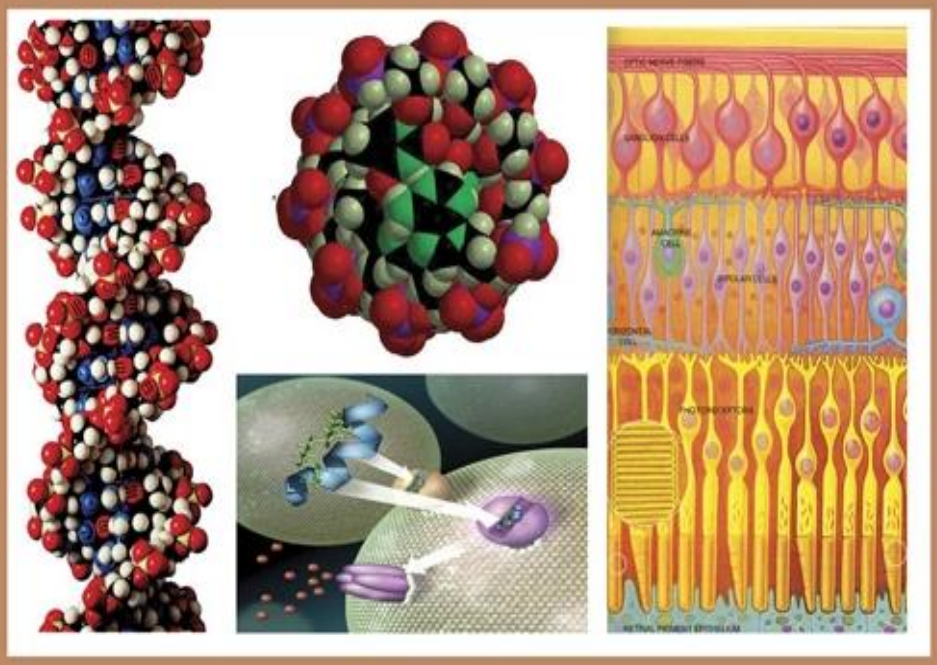

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# Efficiency of Clove Oil Nanoemulsion in Modulating Titanium Dioxide-Induced Some Disorders in the lung of the Mice

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***Keywords***:

$TiO_2$ nanoparticles, lung, clove oil nanoemulsion, DNA fragmentation, antioxidants.

**ABSTRACT**

The goal of the current investigation was to assess the potential therapeutic efficacy of administering clove oil nanoemulsion to treat certain pulmonary problems that male mice's exposure to titanium dioxide nanoparticles (TiO2 NPs) had generated.

It has been demonstrated that titanium dioxide nanoparticles (TiO2 NPs) might have detrimental effects on health and lead to diseases of the respiratory system. Although clove oil possesses anti-inflammatory and antioxidant qualities, its pungent taste, chemical instability, and limited water solubility place restrictions on how much of its potential can be utilized. To get over this limitation, a nanoemulsion of clove oil (NE-CLV) was created**. Material and methods:** Five groups, each with ten mice, were formed from the fifty mature male mice. SOD and GPx are determined as antioxidants and MDA as an indicator of oxidative stress. IL-6 and TNF-α levels were determined in the lung tissue. Genotoxicity was evaluated by using a laddered DNA fragmentation assay. Histopathological examination using hematoxylin and eosin stain. **Results:** SOD and GPx were decreased. While, MDA, IL-6 and TNF-α levels were increased in the injected Titanium dioxide nanoparticles group. Co-administration of the clove oil nanoemulsion ameliorates the changes in these parameters and the histopathological changes. Also, reduced the DNA damage caused by nanotitanium and restored the integrity of the genomic DNA. **Conclusion:** This study's goal was to ascertain whether clove oil nanoemulsion (NE-CLV) could reduce the toxicity of TiO2 NPs by regulating oxidative changes, restoring them to their original state, and preventing genotoxic damage. Therefore, it appears that NE-CLV can be used as a helpful hemoprotective to prevent the toxicity that is brought on by exposure to TiO2 NPs.

## INTRODUCTION

Nanoparticles (NPs) are normally smaller than 100 nanometers and have a higher permeability (Takeuchi *et al.*, 2017). But it could also be dangerous to people's health (Orr *et al.*, 2019).

Titanium dioxide nanoparticles (TiO2 NPs) are largely used in sugar-coated chewing gum, sauces, pastries, cosmetics, cakes, toothpastes, whiten-skim milk confectionery and sunscreens (Zhang *et al.*, 2015). It is also abundant in sweets and food additives found at high concentrations. TiO2 nanoparticles are the most widely utilized NPs due to their dual applications in environmental decontamination and their widespread use in this field (Brun *et al.*, 2014). Due to its extensive use, the detrimental consequences of TiO2 NPs on human health have received increased attention as a result of their widespread use.

It has been demonstrated that nano-titanium is distributed in vivo and accumulates in numerous organs through a variety of exposure modes, including oral, cutaneous, and inhalation (Liu *et al.,* 2009). One of the main ways that people are exposed to nanoparticles in the environment is by inhalation. By diffusion, the inhaled NPs from the Pollutants in the air are stored in the nasopharynx or may enter the lungs (Braakhuis *et al.,* 2014). Because NPs are so reactive, it causes the aqueous environment of biological tissues to produce ROS. By activating multiple defensive mechanisms and inducing inflammation through the activation of inflammatory pathways, the created oxidative stress can overwhelm antioxidant defence systems and cause the release of cytokines (Nel et al., 2006) these are the most obvious biomarker indicators of nanoparticles toxicity (Relier *et al.,* 2017).

Inflammasomes are activated by ROS produced by nanoparticles either directly or indirectly through mitochondrial failure or effectors such as cathepsin B released from damaged lysosomes. Interleukin-activating caspase is then activated (Farrera *et al.,* 2015), Lysosomal damage may result from oxidative membrane damage or poor handling of phagosomes carrying indigestible NPs. Inflammation and tissue damage brought on by NP may potentially be caused by lysosomal leakage and atypical autophagy (Tapsell *et al.,* 2006). The cytoplasmic release of a large number of lysosomal enzymes and aberrant degradation processes can result from cytoskeleton, intracellular transport, and/or lysosome dysfunction (Cohignac *et al.,* 2014).The NPs' surface area, their capacity to generate ROS, and the pro-inflammatory effects brought on by the particles in the lung are all directly correlated (Dudonne et al., 2009). DNA damage and oxidative stress are caused by TiO2 nanoparticles in different organs, including the liver, kidney, spleen, bone marrow, heart, lung, and brain (Xu *et al.,* 2013).

Vegetarian diets include a significant amount of phytochemicals with significant biological activity, such as flavonoids, phenolic acids, etc. (Krishnaswamy *et al.,* 1998). According to Schmidt (1972), among the essential oils is clove oil, which is extracted from the *Syzygium aromaticum* tree and applied topically to relieve pain and speed up recovery. It is also utilised in the flavouring and fragrance sectors. Eugenol represents about 89% of clove essential oil with the remaining 5-15 % consisting of eugenol acetate, cariofileno, and gallic acid. Phenylpropanoids, make up the remainder of the oil (Jirovetz *et al.,* 2006). It possesses anti-inflammatory, antifungal, and antioxidant qualities. It also has local anaesthetic characteristics. (Ghelardini *et al.,* 2009)

## MATERIALS AND METHODS

Fourty male adult mice were obtained from the Dokki-Giza animal house of the national research center. Prior to the experiment, the animals were housed in the lab for at least a week under typical housing conditions (room temperature of 25–27 °Cwith alternate 12-hour cycles of light and darkness). Additionally, they were given atypical meal and water. *ad libitum*. The albino mice were administered a dose of NE-CLV up to 2000 ml/kg body weight (b.w.) (OCED, 2000). But it was observed that the animals were not affected and were still alive after two weeks. Considering that this dose is safe, we administered 100 ml/kg (b.w.) of it. The animals were divided into four groups, with eight in eachgroup, as follows: The first group is the control group, and the second is the NE-CLV group at 100 ml/kg (b.w.). 3rd TiO2 NPs group at 50 mg/kg (b.w.) (Mohamed, 2015). The fourth group wasgiven TiO2 NPs orally at 50 mg/kg (b.w.) followed by 100 mg/kg (b.w.) for five days, after 24 hours from the last dose the mice werekilled and lung samples were collected for

physiological, molecular, and histologicalstudies.

The combination of rutile and anatase-shaped TiO2 nanoparticles used in this study were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA) as an odourless, white powder with a purity of 99.5% and CAS number 13463-67-7. The TiO2 nanoparticles were ultrasonically homogenised in deionized distilled water using a biologics ultrasonic homogenizer before being characterized and administered (Model 150VT). The nano-TiO2 suspensions had a pH of 6.8. X-ray diffraction (XRD) was used to determine the crystal phase and the average crystallite size using the Scherrer's relationship (D 14 0.9 k/Bcosh), where k is the X-wavelength, ray's B is the diffraction line's broadening measured as half of its maximum intensity in radians, and c is the average crystallite size. TiO2 nanoparticles were characterized according to the method mentioned by Mohamed (2015).

**Characterization of the Clove Oil Nanoemulsion (microscopic observations):**

The crude clove oil used in this investigation was purchased from Dr. Ahmed Abo Al-Nasr Center and processed at Naqaa CO. (Cairo-Egypt).

Using the Bouchemal *et al.* (2004) approach, the appearance and structure of the clove oil emulsions were investigated using transmission electron microscopy (TEM). Evaluation of zeta potential (ZP), polydispersity index (PDI) and particle size (PS). Using a Malvern Zetasizer 2000, ZP, PDI, and PS for the created nanovesicles were evaluated (Malvern Instruments Ltd., UK). The calculations were done following the proper dilution (10 folds with de-ionized water). By monitoring the vesicles' electrophoretic movement in the electrical field, the ZP wasevaluated. Triplicates were used for all measurements.

**Transmission Electron Microscopy (TEM):**

The morphology of the perfect vesicles was observed by TEM. (Joel JEM 1230, Tokyo, Japan). On a carbon laminated copper grid, a thin film of one drop of the ideal nanodispersion was placed without dilution and stained with phosphotungstic acid 1.5%.%.

**Laddered DNA Fragmentation Assay Method:**

Based on the procedure given by, the apoptotic DNA fragmentation in the lung tissues was qualitatively evaluated using pulsed field gel electrophoresis (Lee *et al.,* 2010).

1. A little portion of tissue was gently homogenised and lysed in Tris EDTA buffer with 0.5% sodium dodecyl sulphate and RNase A. Samples were incubated at 37°C for one hour.
2. Samples were once more incubated at 50 °C overnight with Proteinase K added.
3. Added one volume of phenol to the genomic DNA extraction process: 25:24:1 isoamyl alcohol and chloroform, vortexed for 20 seconds, centrifuged for 5 minutes at 16000 xg. The layer was carefully transferred to a new Eppendorf. after the overlying aqueous phase was properly removed.
4. The DNA was then precipitated using isopropanol and ammonium acetate.
5. After being electrophoresed in 1% agarose gel at 70 Volts, the isolated DNA was then visualised with a UV transilluminator and photographed.

**Biochemical Parameters:**

**Biomarkers of Oxidative Stress and Antioxidants in Lung Tissue:**

The Nishikimi et al. (1972) method was used to determine the superoxide dismutase (SOD) activity. GPx was estimated utilising the technique of Paglia and Valentine (1967). The levels of MDA were determined according to Ohkawa e*t al.* (1979) procedure at a wavelength of 534nm in the lung tissue homogenate.

**Immunological Parameters:**

TNF-α level in lung homogenate was determined by using RayBio® Rat TNF-alpha enzyme-linked immunosorbent assay (ELISA) according to Brouckaert *et al.* (1993). Estimation of Interleukin-6 (IL-6) level in the lung homogenate by ELISA

*(Bioscience, Austria)*: After homogenising the lung samples in phosphate buffer saline, they were centrifuged at 10,000 rpm for 10 min. Elisa assays were performed on the supernatant.

**Histopathology Study:**

The lungs were then removed and placed in the previously mentioned 4% neutral buffered formaldehyde solution for 24 hours (Poulsen *et al.*, 2016). The samples were cut after fixation and then embedded in paraffin. On a microtome (made by Thermo ScientificTM), sections were cut at 3 m. Hematoxylin and eosin was used to stain sections for light microscopic examination (H&E staining).

**Statistical Analysis:**

Using the computer application SPSS/CP, a statistical package for social sciences, the results were statistically analysed (version 20). ANOVA (one-way analysis of variance) was used to examine the results. The mean SE was used to express all values. Differences were considered statistically significant at ($p < 0.05$).

## RESULTS

**Nanovesicle Characterization:**

Evaluation of PS, PDI, and ZP. Thegenerated vesicles' PS, which measured 218.1±2.27 nm, indicated that they were in the nanoscale. In terms of PDI, A population with a value of zero is entirely symmetric, while a population with a value of one is entirely polydisperse. The generated nanovesicles' PDI of 0.31±0.01 indicated that they were homogenous with a narrow size distribution. The produced nanovesicles had good stability as evidenced by the ZP potential measurements, which showed an extremely negative value of -21.6±0.50mV.

**Transmission Electron Microscopy (TEM)**:

Using TEM examination, the exterior morphology of the nanovesicles was investigated. Nanovesicles' morphological study revealed that they had a homogeneous size distribution and a spherical shape (Fig. 1). Zetasizer's PS estimate for the vesicles was in good agreement with the findings of TEM.

The electrophoresed genomic DNA's pattern on 1% agarose of lung tissues of the negative control (C), clove oil nanoemulsion group (NE), titanium dioxide nanoparticles (Tnps) and titanium dioxide nanoparticles plus nanoemulsion (Tnps plus NE) groups. Laddered DNA fragmentation assay (Fig. 2).

Oral administration of the clove oil-nanoemulsion (NE) alone did not cause DNA damage as indicated by the intact manifestation of genomic DNA.However, the integrity of genomic DNA was severely disrupted in lung tissues from mice given nanoparticles of titanium dioxide (NT) as seen by the genomic DNA's extremely fragmented appearance on an agarose gel in contrast to the pattern of intact negative control genomic DNA.

The intact appearance of the genomic DNA of mice given the clove oil nanoemulsion (NE) simultaneously with titanium dioxide nanoparticles (NT) demonstrated that co-administration of the clove oil nanoemulsion (NE) decreased NT-induced DNA damage and improved genomic DNA integrity.

The current study discovered a significant ($p < 0.05$) decline in SOD and GPx activity in the lung tissue followinginjection of nano-TiO2. But MDA-content increased versus the control group. While combining NE-CLV oil oral dose with TiO2 NPs results in a significant ($p < 0.05$) improvement in these alterations versus the $TiO_2$ NPs group (Figs. 3 A, B and C). The injection of TiO2 NPs causes a significant ($p < 0.05$) increase in IL-6 and TNF levels in the lung tissue when compared to control. In contrast to the TiO2 NPs group, treating with NE-CLV oil concurrently with TiO2 NPs significantly ($p < 0.05$) improved IL-6 and TNF- levels (Figs. 4A and B).

The toxic group that is administered with TiO2 NPs shows thickening of the interstitial tissue and congestion of the peribronchial blood vessel in the lung tissue stained with hematoxylin and eosin. While the protective toxic group (NE-CLV and TiO2 NPs) indicated regression in the interstitial tissue thickening (Fig. 5).

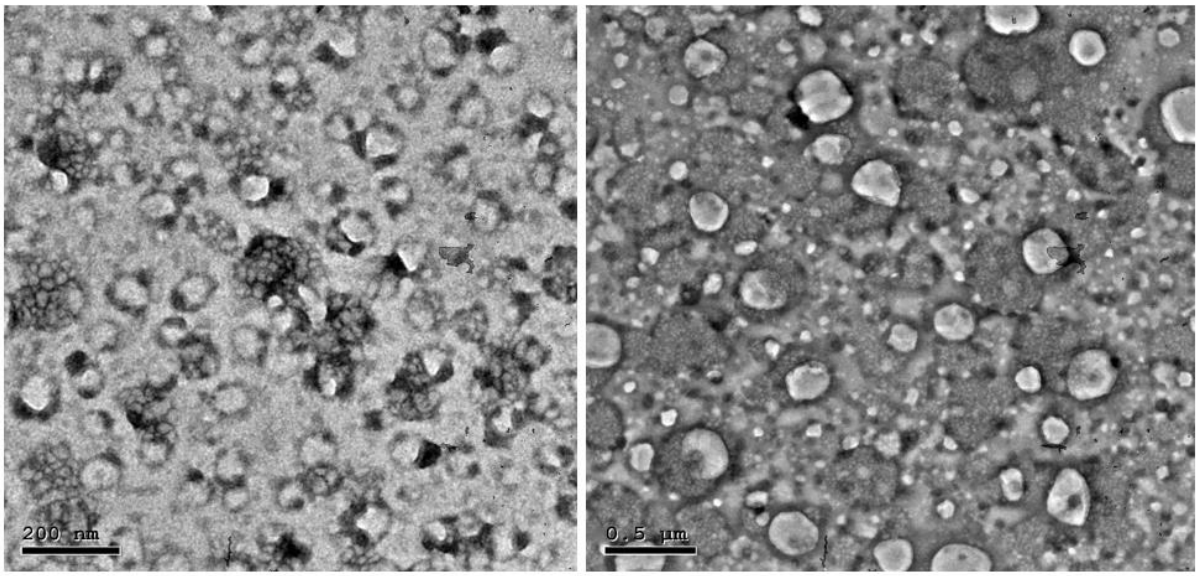



**Fig. 1:** Morphology of clove oil nanoemulsion.

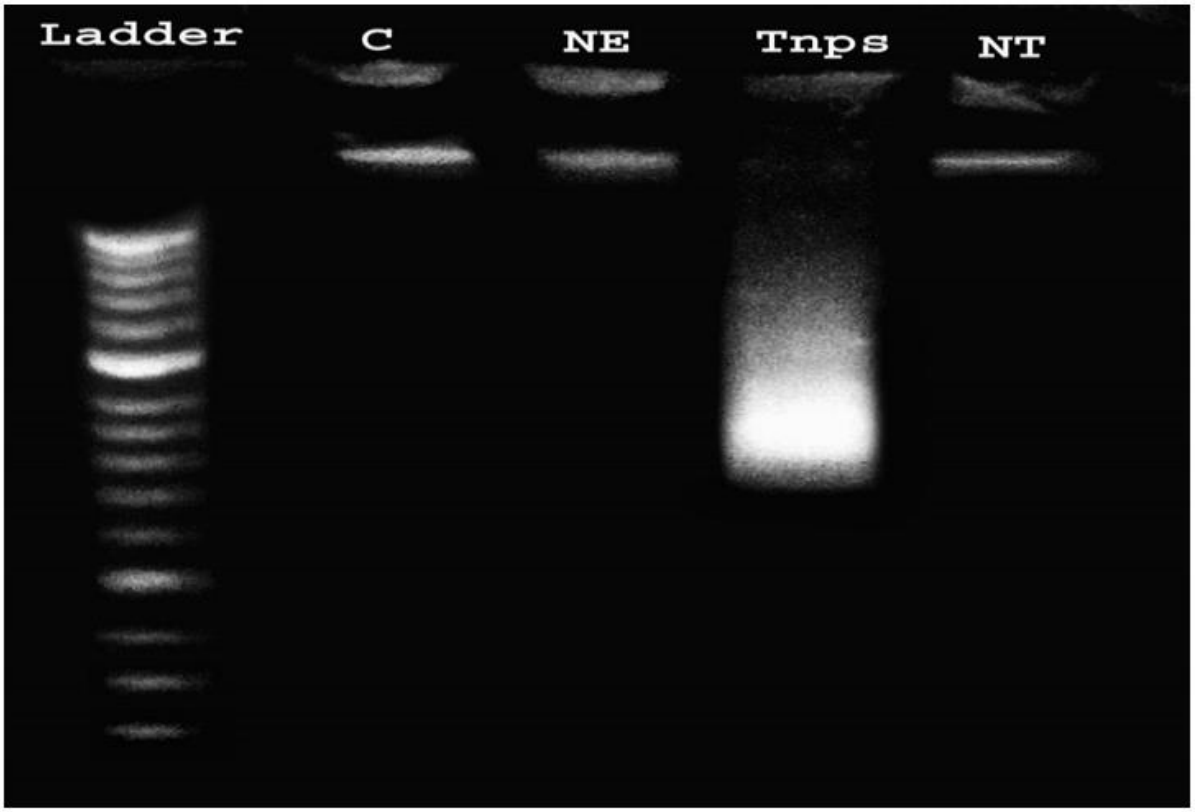
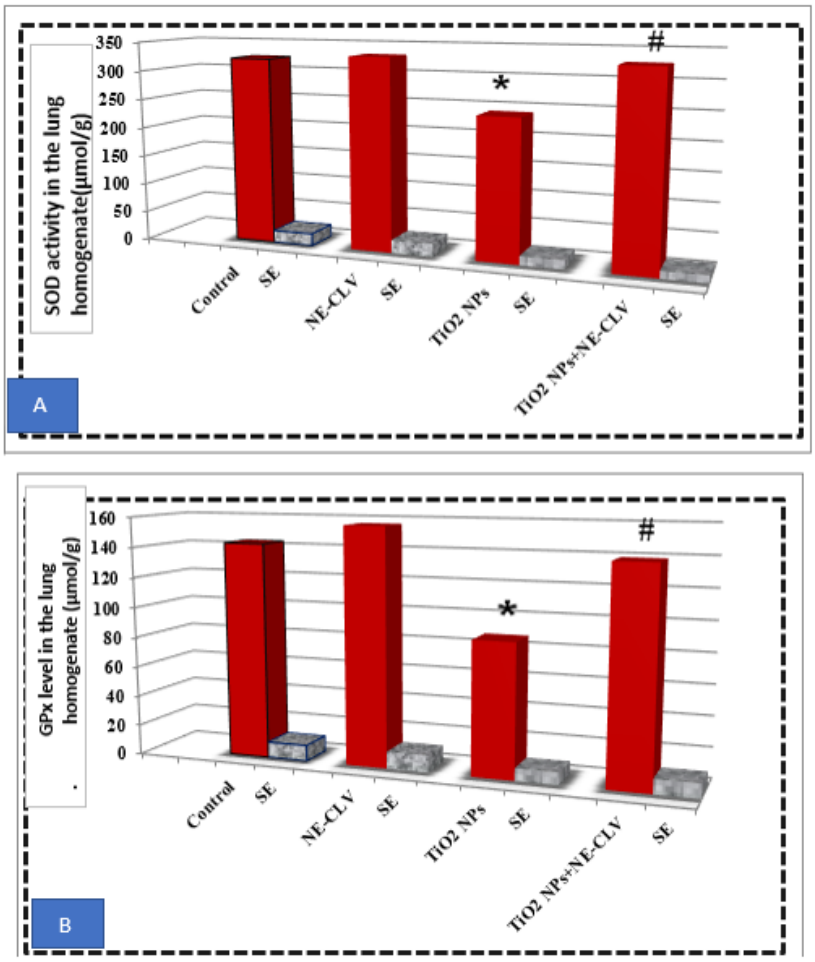



**Fig. 2.** Result of the Laddered DNA fragmentation assay.




**Figs. 3A and 3B.** Effect of clove oil-nanoemulsion (NE-CLV) against titanium oxide nanoparticles (TiO2 NPs)-induced alterations in SOD (**Fig. 3A**) and GPX activity (**Fig. 3B**) in the lung tissue homogenate (µmol/g). Each bar with a vertical line and the nearest small bar represents the mean ± SE. * Significant ($p < 0.05$) versus the control, # Significant ($p < 0.05$) vs the $TiO^2$ NPs group.

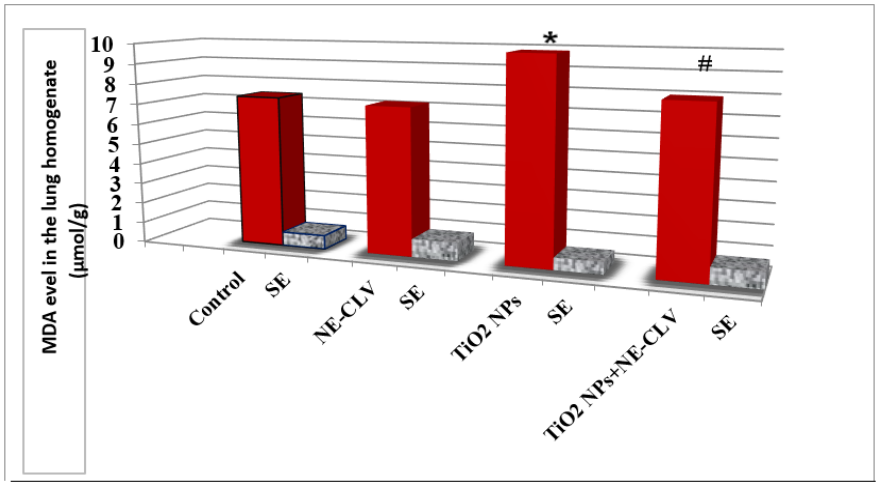



**Fig. 3C.** Effect of clove oil-nanoemulsion (NE-CLV) against titanium oxide nanoparticles -induced alterations in MDA content in lung tissue homogenate (µmol/g). Each bar with a vertical line and the nearest small bar represents the mean ± SE. * Significant ($p < 0.05$) in comparison with the control group, # Significant ($p < 0.05$) vs the TiO2 NPs group.

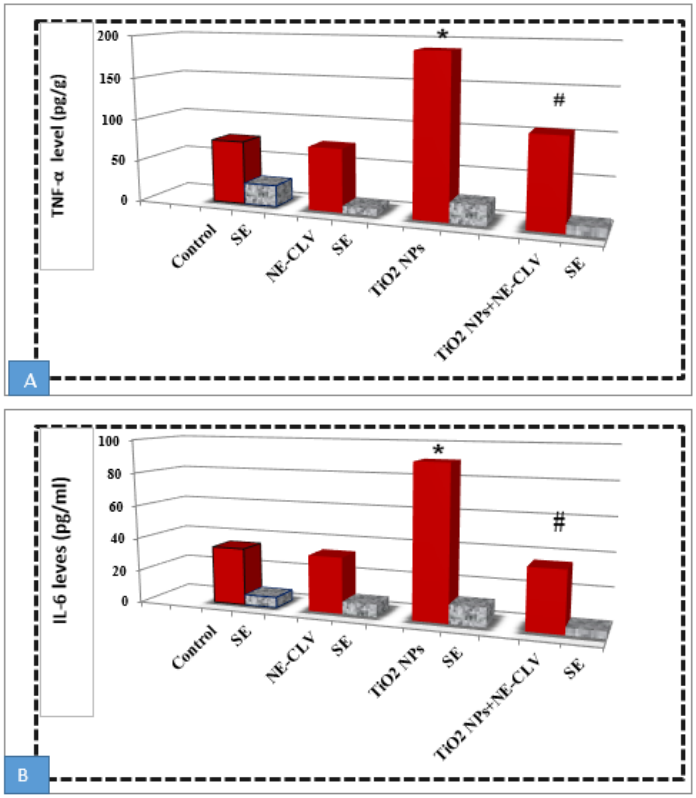



**Fig. 4A and 4B** Effect of clove oil-nanoemulsion (NE-CLV) against titanium oxide nanoparticles (TiO2 NPs)-induced alterations in **TNF-α and IL-6 levels** in lung tissue homogenate (µmol/g). Each bar with the vertical line and the nearest small bar represents mean ± SE. * Significant ($p < 0.05$) when compared to the control group, # Significant ($p < 0.05$) in comparison with the TiO2 NPs group. NE-CLV, clove oil nanoemulsion; TiO2 NPs, titanium oxide nanoparticles.

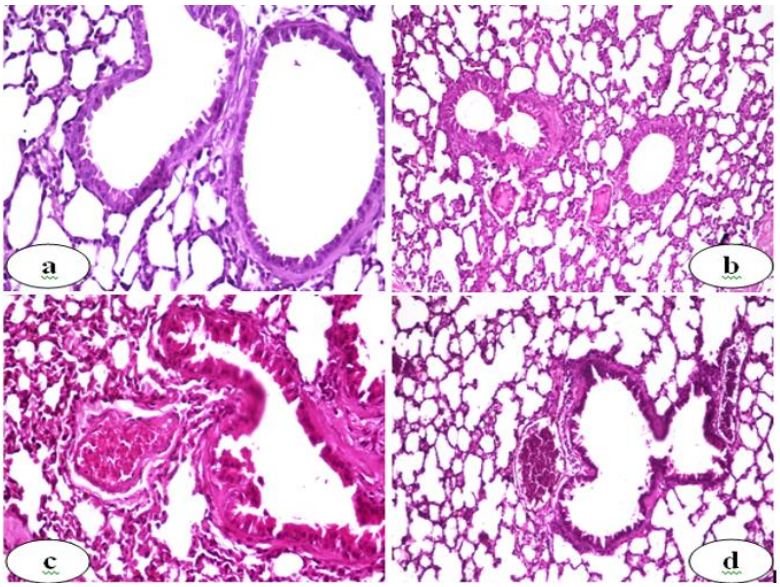



**Fig. 5.** Hematoxylin and Eosin-stained photomicrographs of the lungs from several experimental groups are displayed**. (a)** Control negative group with normal lung parenchyma; note the normal bronchi and alveoli (X200). **(b)**Clove oil nanoemulsion (NE-CLV) group with apparently healthy lung bronchi and alveoli (X200). **(c)**The toxic group that is administered with (TiO2 NPs) shows thickening of the interstitial tissue and congestion of the peribronchial blood vessel (X400). **(d)** Protective toxic group (NE-CLV) and (TiO2 NPs) with regression in the interstitial tissue thickening (X200).

## DISCUSSION

This study's goal was to determine how nanoemulsion of clove oil (NE-CLV) may protect against lung damage brought on by nanoparticle-TiO2 (TiO2 NPs)

With nanotechnology's recent, rapid advancement, Nano-TiO2 has found widespread application in a variety of fields, including coatings, paints, cosmetics, food, and others (Wani *et al.,* 2021). The National Institute for Occupational Safety and Health has designated nano-TiO2 as a chemical that may cause cancer (Stapleton *et al.,* 2018). However, the health issues raised by exposure to TiO2 NP remain to be resolved.

It was demonstrated that oxidative bronchial epithelial cell DNA damage might be caused by ultrafine TiO2 NP particles (Hart and Hesterberg, 1998).
Additionally, TiO2 has the potential to break DNA double-strands by continuously producing more ROS (Msiska *et al.,* 2010).

The oxidative stress indicators in exhaled breath condensate samples were significantly higher in TiO2 manufacturing employees compared to unexposed controls (Pelclova *et al.,* 2016).

The substantial increases in MDA levels resulted in oxidative stress by depleting cellular GSH and resisting the protective actions of cellular antioxidant enzymes like SOD and GPx, which can induce lipid peroxidation and injure cells, suggest that nano-TiO2-induced genotoxicity was caused by the accumulation of ROS produced by TiO2 NP.

The mechanism that has been hypothesised, according to Wang *et al.* (2009), involves ROS production as a defining feature of TiO2-NP toxicity. TiO2-NPs have noticeably increased pulmonary inflammatory effects. (Sun *et al.,* 2012) explored how intratracheal injection of TiO2 NPs with increasing exposure time dramatically boosted ROS generation (elevated O2, H2O2) in mouse lungs. In order to adjust intracellular responses to TiO2-induced oxidative stress, treatment with TiO2 NPs resulted in extracellular ROS production and elevated TNF- release (Yazdi 2010).

The current investigation found decreases in SOD and GPx activities in lung tissues after injection of nano -$TiO_2$.While MDA content was increased. This is in line with the conclusions of Yogalakshmi *et al.* (2010) and Han et al. (2020). While treatment with NE-CLV ameliorates these changes. Theseresults are in line with those of Zin *et al.* (2012), who found that the clove oil component eugenol significantly contributes to the preservation of lung tissue against changes in MDA content.

Chronic inflammatory disorders like asthma, atherosclerosis, and inflammatory bowel disease (Netea et al., 2017) may occur as a result of improper control of inflammatory reactions. Immunodeficiencies can also be caused by immune system parts that malfunction as a result of genetic flaws that are passed down through families or harm from environmental causes like poor nutrition or drugs (Marshall *et al.,* 2018)

TiO2-NP injection causes an increase in IL-6 and TNF- levels in lung tissue, this is in line with Relier *et al.* (2017) and Chen etal. (2018) respectively. While treatment with clove oil-nanoemulsion caused amelioration of these changes, this agrees with the findings of Yogalakshmi *et al.* (2010). Theydiscovered improvements in antioxidant status, including GPx & SOD and a reductionin TNF- and IL-6 production.

Clove oil (CO) is enriched with many antioxidant compounds (Ogata et al., 2000). Due to the presence of eugenol, CO has a protective effect against lipid peroxidation and has an anti-genotoxic impact (Sharma *et al.,* 2011). Additionally, its antioxidant characteristics significantly protect DNA (Jayakumar and Kanthimathi, 2012).

This study showed that administration of TiO2 NPs causes thickening of the interstitial tissue and congestion of the peribronchial blood vessel. This agrees with the results of Horvath *et al.* (2018) who discovered hyperplasia of the interstitium or alveolar epithelium and macrophage growth in the alveolar area of rat lungs.

## Conclusions

There are many advantages and potential hazards associated with nanoparticles for human health due to their small size and distinct physical and chemical characteristics. TiO2 is widely utilised in various sectors and is thought to be low toxicity. However, at the nanoscale, rats' lungs and extrapulmonary organs could accumulate TiO2, which would then induce oxidative stress, which would ultimately result in DNA damage. All these alterations were less pronounced in the group that received TiO2 NPs and clove oil nanoemulsion (NE-CLV). Therefore, it would appear that NE-CLV can be employed as a chemoprotective agent against the toxicity caused by TiO2 NPs.

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