



Influence of Micronutrients in The Alleviation of Immunotoxicity Induced by Poly Aromatic Hydrocarbons Naphthalene and Anthracene

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Abstract: Diet plays a major role in fine tuning the immune system and to combat against dreadful diseases and infections. Micronutrients form an important part of balanced diet and enable efficient functioning of the immune system. Vitamins, minerals and trace elements comprise the major category of micronutrients. Deficiency of micronutrients could culminate in suppression of both adaptive and innate immunity leading to altered immune homeostasis. Anthracene and naphthalene are two environmental pollutants which are highly toxic, carcinogenic poly cyclic aromatic hydrocarbons that gains entry in to the human system through multiple modes. The aim of the current study is to evaluate the potential of four dietary micronutrients Vitamin A, Vitamin C, Folic acid and Zinc to counteract the immunotoxic effects of naphthalene and anthracene on Murine macrophage cell line RAW 264.7. Several cell based assays like MTT assay, LDH leakage assay, Crystal violet test, DCFDA analysis (for ROS generation) and Rhodamine B assay (for measurement of mitochondrial membrane potential) were taken as end point indicators to support the findings of the study. The results showed that, both naphthalene and anthracene triggered the production of ROS, altered mitochondrial membrane potential, and induced strong immunosuppressive effects. The immunosuppressive effect was found to be more prevalent in anthracene treated cells. Pre-treatment with the micronutrients exhibited considerable protection against naphthalene and anthracene induced changes in the cells primarily through the antioxidant and immunomodulatory effects. The overall protective effects offered against naphthalene and anthracene was found to be superior in the Vitamin A treated cells as compared Vitamin C, Folic acid and Zinc treated cells. Prolonged immunosuppression reduces immune surveillance and hence could activate oncogenic signals leading to carcinogenic conditions. Regular micronutrient supplementation in the diet could help considerably to negate or prevent these deleterious conditions.

Key Words: Micronutrients, Anthracene, Naphthalene, PAH, Macrophages, RAW 264.7.

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1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) represent an important class of environmental pollutants, some of which have been shown to be suppressive to the immune system of humans and several animal species ¹. They are ubiquitous group of several hundred chemically related compounds, that are recalcitrant in nature, with various structures and varied toxicity. The compounds that chemically consist exclusively of hydrogen and carbon atoms are referred to as "PAH". Chemically PAHs are comprised of two or more benzene rings bonded in linear, cluster or angular arrangements ². Naphthalene is an organic compound with formula C₁₀H₈. It is a white crystalline solid with a characteristic odour and is a major component of moth balls used as insect repellants. The compound has two fused benzene rings ³ which makes the compound recalcitrant in nature. The International Agency for Research on Cancer (IARC) (vide IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, December 25 2008 has classified naphthalene as a Group 2 B carcinogen ("possible carcinogen") to humans and animals (Group 2B) ⁴. Acute exposure to the compound could induce formation of cataracts in mice, rats, rabbits and humans. Maternal exposure to the compound during pregnancy or oral/inhalation toxicity could result in haemolytic anaemia in infants and children. Anthracene is also a polycyclic aromatic hydrocarbon which structurally comprises three benzene rings that are fused together. The compound has a molecular formula of C₁₄H₁₀, and like many other polycyclic aromatic hydrocarbons, is generated during combustion processes. Human exposure to anthracene is predominantly through consumption of food, comprising high concentration of combustion products (smoking, grilling, barbecuing, high temperature baking, roasting, frying etc.,) or through exposure to tobacco smoke⁵. Thus EPA (Environmental Protection Agency, 2008) ⁶ has classified anthracene as probable human carcinogen. Damage to cells caused by environmental pollutants and toxicants is believed to play a central role in disease progression. The immune system comprises a delicate and highly complex network of cells, and organs that functions to recognize and eliminate foreign invaders of the body. The primary function of the immune system is to defend human beings, non-human primates and other vertebrates against the attack of bacteria, viruses, parasites and fungi. Effective nutritional interventions in the immune system may find value not only in therapeutic applications, but also in the prophylactic treatment of subjects at risk of immune incompetence. Prevalence of pre-existing illness (AIDS, Tuberculosis), immunosuppressive treatment regimes (organ transplant recipients, cancer patients on chemotherapy/radiation therapy) and complex surgical procedures, could all compromise immunity and alter immune homeostasis. Nutrient status is an important factor contributing to immune competence and the profound interactions between nutrition, infection, and health were recognized. Lymphocytes are the principal mediators of the adaptive immune response ⁷. To evoke an immune response, effective antigen presentation by professional antigen presenting cells like macrophages and dendritic cells are needed. Macrophages are cells that play an important role in innate immunity as well as adaptive immunity. In innate immunity, they form a first line of defense and perform antigen capture by phagocytosis. In adaptive immunity, macrophages act as antigen presenting cells, and play an indispensable role in the outcome of effective immune responses. The efficient functioning of the immune system and the outcome of immune response is determined by adequate nutrient intake,

especially micronutrients like vitamins, trace elements and minerals. Deficiency of micronutrients in the diet is reported to suppress both, innate as well as adaptive immune responses culminating in altered immune homeostasis. Breach of barriers, reduced humoral immunity and defective antigen recognition and response could all result from micronutrient deficiency. Both the water soluble and lipid soluble vitamins together with trace elements like zinc, selenium and copper work in tandem with each other to enable the efficient functioning of the immune system. In short, the health of the immune system and overall well being of the individual is closely related to the regular, balanced supplementation of the micronutrients in the diet. Therefore, supplementation with these selected micronutrients can support the body's natural defence system by enhancing all three levels of immunity⁸. The objective of the current study is to evaluate the efficacy of some micronutrients, to negate the immunotoxicity induced by poly aromatic hydrocarbons naphthalene and anthracene on murine macrophage cells RAW 264.7. As macrophages are cells involved primarily in antigen capture and antigen presentation, they are actively involved in innate as well as adaptive immunity. Hence the study was conducted on macrophage cells as in vitro model systems.

2. MATERIALS AND METHODS

2.1 Procurement, establishment and maintenance of Murine macrophage cell line RAW 264.7

Murine macrophages RAW 264.7 cells were procured from National Center for Cell Sciences (NCCS), Pune, India. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with IX antibiotic antimycotic solution and 10% fetal bovine serum (FBS; Himedia, India). Cells were grown under standard growth conditions (temperature 37° C, 5% CO₂ and 95% humidity) in a CO₂ incubator (Forma Scientific, USA) ⁹. When a confluent monolayer was formed, cells were detached with a cell scraper (Himedia, India) in Dulbecco's phosphate buffered saline (Himedia, India) and then subcultured at a split ratio of 1:3 in a tissue culture flask of about 12.5 cm volume. The media was changed every alternate day. Growth media or maintenance media was used as per the requirement for the assays The growth medium contained 10% FBS whereas the maintenance medium contained about 5% FBS. After arriving at confluency, the cells were seeded on to 96 well microtitre plates (Himedia, India) and were utilized for various assays.

2.2 Assays performed

2.2.1. Segregation of experimental groups and preliminary studies for dose fixation:

Briefly, 5 × 10⁴ cells/ml were cultured in 96 well plate overnight and pre-treated with different concentrations of Vitamin A (25, 50, 75 µg/ml), Vitamin C (50, 100 and 150µg/ml), Folic acid (25, 50, 75 µg/ml) and Zinc (15, 25, 35 µg/ml) respectively for 30 minutes. Following pre-treatment with the test compounds the cells were exposed to naphthalene (300µM) and Anthracene (500 µM) respectively. These two doses were fixed after preliminary studies with three different concentrations of naphthalene (300, 500 and 700µM) and anthracene (150, 300 and 450µM) on macrophage cells. The untreated cells served as control. Ic₅₀ values were determined by MTT assay as described below and all further assays were conducted with the same. Three replicates were

kept for each well and the experiment was repeated on at least three different occasions. The mean of the replicate values were taken for the determination of IC_{50} values.

2.2.2. MTT assay

The assay was performed following the method of Mossman, 1983¹⁰. Cells were allowed to grow for 24 hours (temperature 37°C, 5% CO₂ and 95% humidity). After the completion of 24 hrs, 25 µl of MTT (10mg/ml in PBS) was added to the wells and the plates were incubated for 4 hours. The formazan crystals formed were solubilized by adding 75µl of Di-methyl Sulphoxide (DMSO) and the plates were read at 570nm using a microplate reader. The doses which gave the maximum protection against immunotoxicity induced by naphthalene and anthracene were used for further studies like crystal violet test, mitotic index assay, DCFDA analysis and analysis of mitochondrial membrane potential.

2.2.3 Crystal violet test

To determine the cell viability, crystal violet assay was carried out following the method of Lena et al, 2009¹¹. The cells were plated and pretreated with IC_{50} doses of naphthalene and anthracene and then treated with optimal doses of vitamin A, Vitamin C, folic acid, and zinc (described in section 2.2.1). After 24 hours of incubation (temperature 37°C, 5% CO₂ and 95% humidity) cells were fixed with 10% formalin saline for 30 minutes (50 µl was added to each well), then the cells were stained with crystal violet (0.05% w/v) for 30 minutes. After that, the wells were washed thoroughly with distilled water to remove the unbound dye (images were captured) and destained with Sorenson's buffer (0.1 M sodium citrate in 50 % ethanol, pH 4.2). The absorbance at 540nm was determined by using a microplate reader (Bio Rad, India).

2.2.4. LDH cytotoxicity assay

The release of lactate dehydrogenase from treated and untreated (control) cells was determined using LDH-cytotoxicity colorimetric Assay kit (Biovision, catalog no.K313-500) as per the manufacturer's recommendations. After the desired treatment period of the cells, 10 µl of the medium (which is clear) was aspirated from different groups (Control and treated) and added to the wells of a 96 well microtiter plate. Following this 100 µl of LDH reaction mixture was added to the wells. The plate was subjected to incubation at room temperature for 30 minutes. Upon the completion of incubation period, 10 µl of stop solution was added to each well to arrest the reaction. The intensity of red colour developed which is proportional to the activity of LDH was determined by measuring the absorbance in a microplate reader at 450nm.

2.2.5. Measurement of intracellular ROS generation [2',7' Dichlorofluorescein Diacetate (DCFDA) assay]

DCFH-DA is the most widely used probe for detecting intracellular oxidative stress. The measurement of intracellular ROS formation is based on the oxidative conversion of 2',7'-dichlorofluorescein-diacetate (DCFH-DA) to fluorescent compound dichlorofluorescein (DCF) (Lin et al., 2006¹²; Johnson 1980¹³). After treatment, cells were harvested and suspended in 0.5 ml PBS containing 10µl DCFH-DA for 15 min at 37°C in the dark. DCFH-DA was taken up by the cells and deacetylated by cellular esterase to form a non-

fluorescent product 2,7-dichlorodihydrofluorescein (DCFH). During the generation of ROS the non-fluorescent product DCFH is converted to a green fluorescent product 2',7' - dichloro fluorescein (DCF). The intensity of the fluorescence (excitation and emission at 488 and 530 nm) in the treated and control cells was measured using a spectro fluorimeter which is proportional to the measure of the severity of oxidative stress induced by ROS generation.

2.2.6. Mitochondrial membrane potential (Rhodamine B assay).

Rhodamine B is used as a mitochondrial probe for measurement and monitoring of mitochondrial membrane potential in drug-sensitive and -resistant cells. Rhodamine derivative R123 is a cationic membrane permeable fluorescent dyes that are readily sequestered by active mitochondria without cytotoxic effects. Mitochondrial membrane potential of treated and untreated control cells was measured using rhodamine 123 (RH123) following the protocol described previously [Desagher et al., 2000¹⁴]. Approximately 1×10^6 cells (both treated and untreated control) was resuspended in 1ml of culture medium following which 2µl RH123 (10µg/ml) was added. The intensity of the fluorescence (excitation and emission at 502 and 530 nm) in the different wells was measured with a spectrofluorimeter.

2.3. Statistical analysis

All experiments were carried out in triplicate on at least three different occasions and the mean of replicate values were taken. Values were expressed as mean \pm SD. Statistical analysis of the data was determined by Student's t-test and comparisons were made between the control and test groups.

3. RESULTS AND DISCUSSION

Nutrient availability has the potential to affect all aspects of the immune system. In general, deficiency of several nutrients will lead to impaired immune responses, and replenishment of those specific components will typically restore the affected responses¹⁵. Polycyclic aromatic hydrocarbons (PAHs) are an important class of environmentally prevalent xenobiotics that cause complex effects on the immune system¹⁶. They are formed by pyrolysis or incomplete combustion of organic materials such as coke, coal tar and pitch, asphalt, and oil¹⁷. Owing to the potent carcinogenic effects of these compounds on humans, there has been an upsurge in the research interest with a special emphasis on immunotoxicity inflicted on animals and human beings. It was suggested that there is a strong correlation between the carcinogenicity of PAHs, their immunotoxicity and perhaps other diseases¹⁸. These agents have been shown to alter antigen and mitogen receptor signaling pathways, leading to suppression of humoral and cell-mediated immunity. Therefore, it is likely that given sufficient levels of exposure to PAHs, humans may be immunosuppressed by these agents¹⁹. In the current study, four micronutrients Vitamin A, Vitamin C, Folic acid and Zinc which are well known for their various beneficial effects on human health were investigated for their ability to counteract the toxicity induced by two potent poly aromatic hydrocarbons naphthalene and anthracene.

3.1 Effect of micronutrients on the growth of macrophage cells during immunotoxicity induced by naphthalene and anthracene.

Figures 1 and 2 shows the effect of different doses of naphthalene and anthracene respectively, on the proliferation of macrophages in culture. A dose dependent decrease in proliferation of macrophage cells was observed on treatment of the cells with both naphthalene and anthracene. Out of the three different doses tested for naphthalene (300, 500 and 700 μ M) and anthracene (150,300 and 450 μ M), it was observed that approximately 50% death of the cells was observed at a dose of 500 μ M for naphthalene and 300 μ M for anthracene. Hence, these doses were accepted as the IC_{50} for naphthalene and anthracene respectively and used for all the other assays carried out in the study. Figures 3, 4, 5 and 6 show the effects of pretreatment with micronutrients Vitamin A, Vitamin C, Folic acid and Zinc respectively against naphthalene induced immunotoxicity whereas Figures 7, 8, 9 and 10 show the effects of pretreatment with the micronutrients against anthracene induced immunotoxicity on murine macrophage cells cultured *in vitro*. Different concentrations of the micronutrients were used in the assay Viz., Vitamin A (25, 50 and 75, μ g/ml), Vitamin C (50, 100 and 150 μ g/ml), Folic acid (25, 50 and 75, μ g/ml) and Zinc (15, 25 and 35 μ g/ml) against the IC_{50} concentrations of naphthalene and anthracene. It was observed that Vitamin A at a concentration of 75 μ g/ml, Vitamin C at a concentration of 150 μ g/ml , Folic acid at a

concentration of 75 μ g/ml and Zinc at a concentration of 35 μ g/ml were able to protect against the toxic effects of both naphthalene (500 μ M concentration) and anthracene (300 μ M concentration). The changes observed were found to be statistically significant ($P < 0.001$). These results highlight two findings. One is that, the intensity of damage inflicted by anthracene on the macrophages is appreciably higher than that inflicted by naphthalene. Hence, anthracene is a more potent immunotoxic PAH compared to naphthalene in humans. This could be the reason as to why a lower dose of anthracene (300 μ M) was sufficient to bring down the cell viability by 50% compared to naphthalene (500 μ M). These results are in agreement with previous reports on the strong immunosuppressive effects of polyaromatic hydrocarbons^{20, 21}. The second finding is that almost the same concentration of each of the compounds were able to confer protection against cytotoxicity induced by both naphthalene and anthracene. The results implicate that the protective effects offered by the micronutrients (Vitamin A, Vitamin C, Folic acid and Zinc) does not differ with respect to naphthalene and anthracene. It is possible that the compounds (micronutrients) have a common detoxification mechanism for naphthalene and anthracene. This is in tandem with reports on the protective effects of micronutrients on the immune system²² and to prevent immunotoxicity²³.

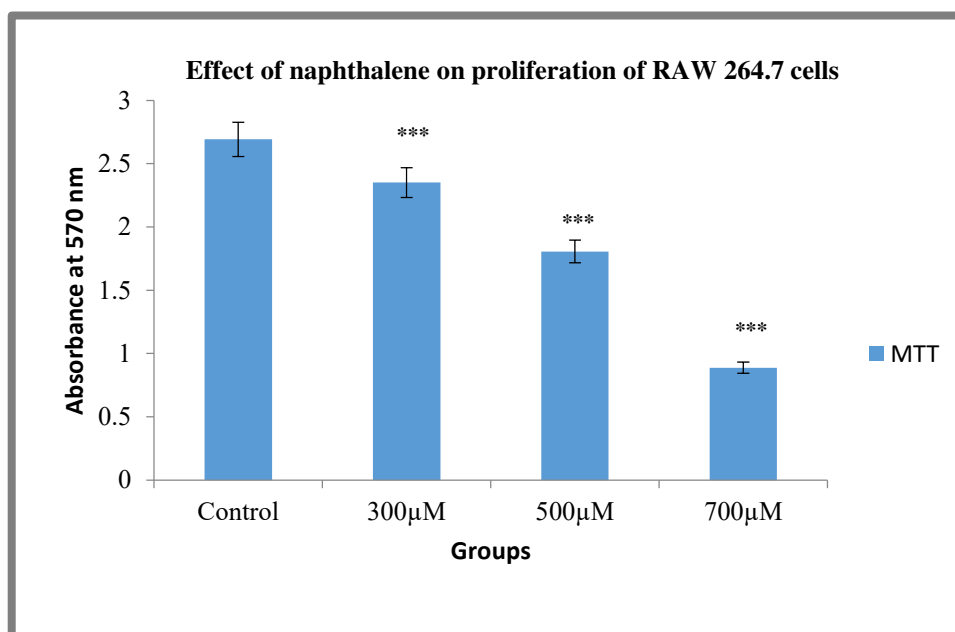


Fig 1: Preliminary studies for the determination of toxicity of naphthalene

Values were expressed as mean \pm SD. Statistical analysis by Students-t test. Comparisons were made between untreated control Vs treated groups. *** $p < 0.001$

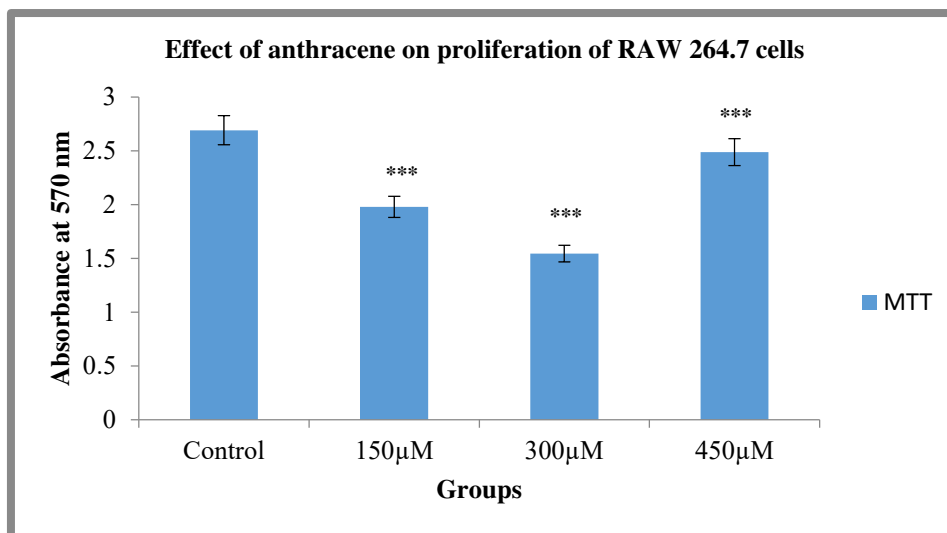


Fig 2: Preliminary studies for the determination of toxicity of anthracene

Values were expressed as mean \pm SD. Statistical analysis by Students-t test. Comparisons were made between untreated control Vs treated groups. *** $p < 0.001$

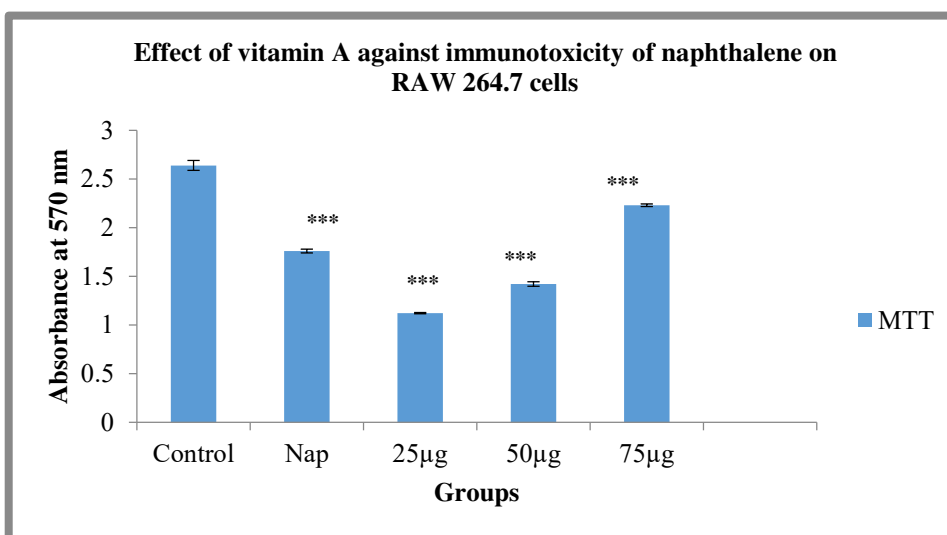


Fig 3: Protective effects of Vitamin A against naphthalene induced toxicity -MTT assay

Values were expressed as mean \pm SD. Statistical analysis by Students-t test. Comparisons were made between untreated control Vs treated groups. *** $p < 0.001$

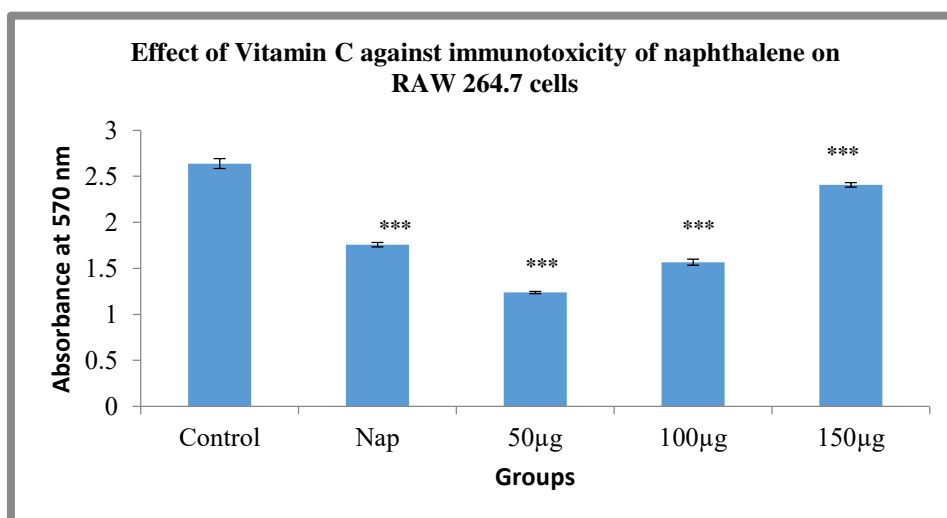


Fig 4: Protective effects of Vitamin C against naphthalene induced toxicity -MTT assay

Values were expressed as mean \pm SD. Statistical analysis by Students-t test. Comparisons were made between untreated control Vs treated groups. *** $p < 0.001$

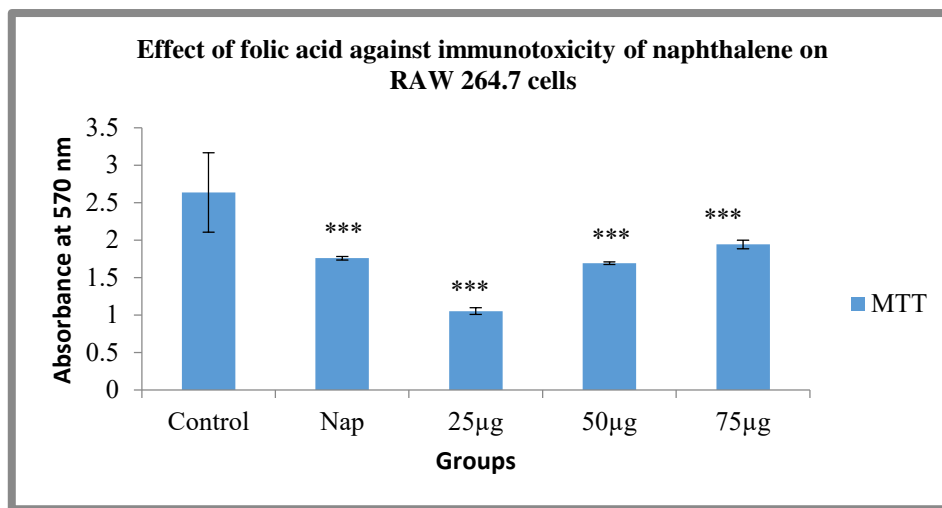


Fig 5: Protective effects of Folic acid against naphthalene induced toxicity -MTT assay

Values were expressed as mean \pm SD. Statistical analysis by Students-t test. Comparisons were made between untreated control Vs treated groups. *** $p < 0.001$

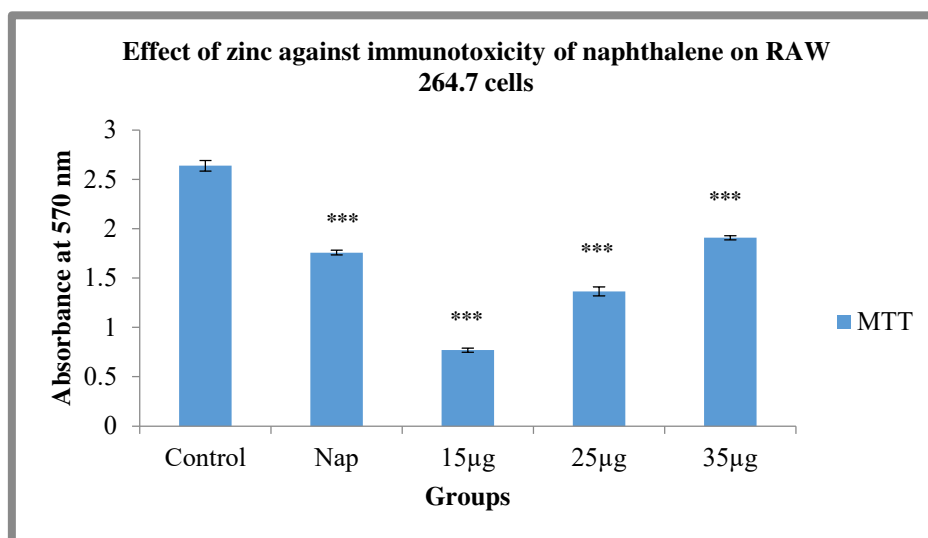


Fig 6: Protective effects of Zinc against naphthalene induced toxicity- MTT assay

Values were expressed as mean \pm SD. Statistical analysis by Students-t test. Comparisons were made between untreated control Vs treated groups. *** $p < 0.001$

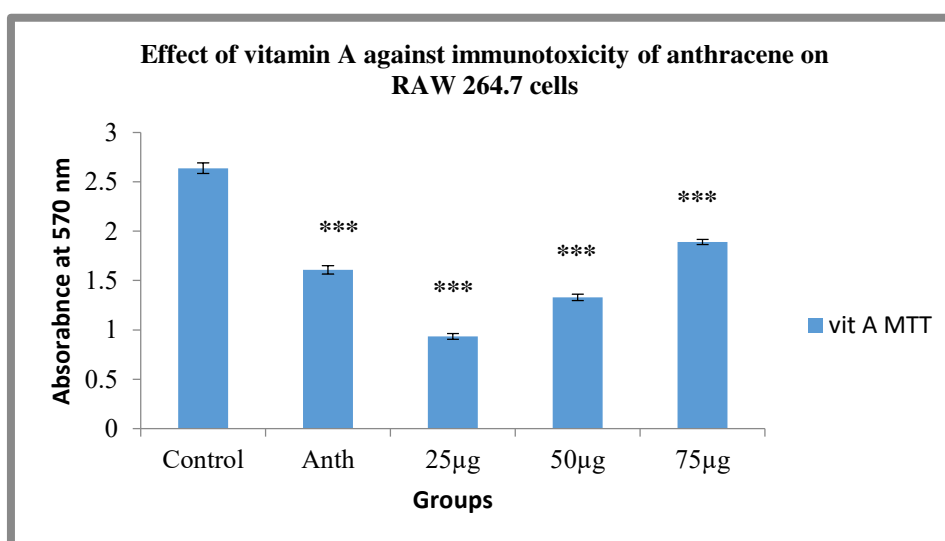


Fig 7: Protective effects of Vitamin A against anthracene induced toxicity -MTT assay

Values were expressed as mean \pm SD. Statistical analysis by Students-t test. Comparisons were made between untreated control Vs treated groups. *** $p < 0.001$

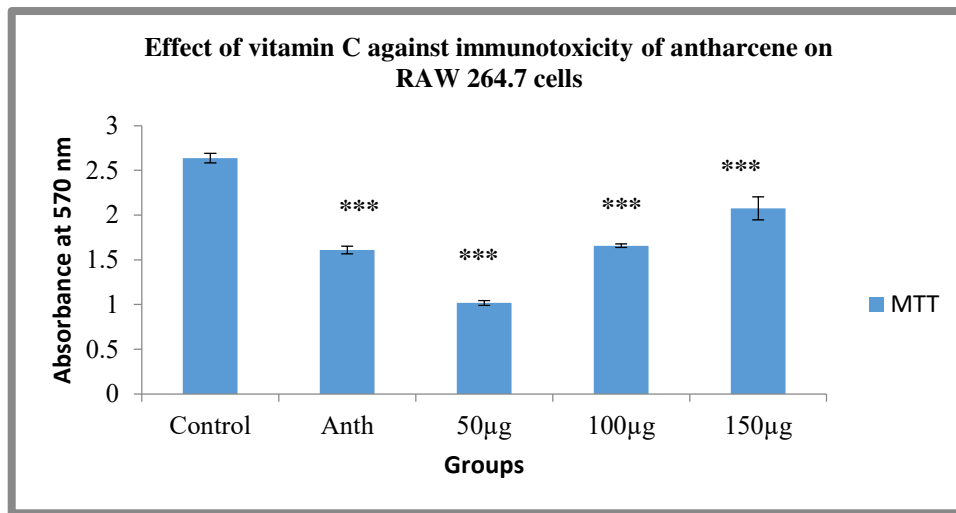


Fig 8: Protective effects of Vitamin C against anthracene induced toxicity - MTT assay

Values were expressed as mean \pm SD. Statistical analysis by Students-t test. Comparisons were made between untreated control Vs treated groups. *** $p < 0.001$

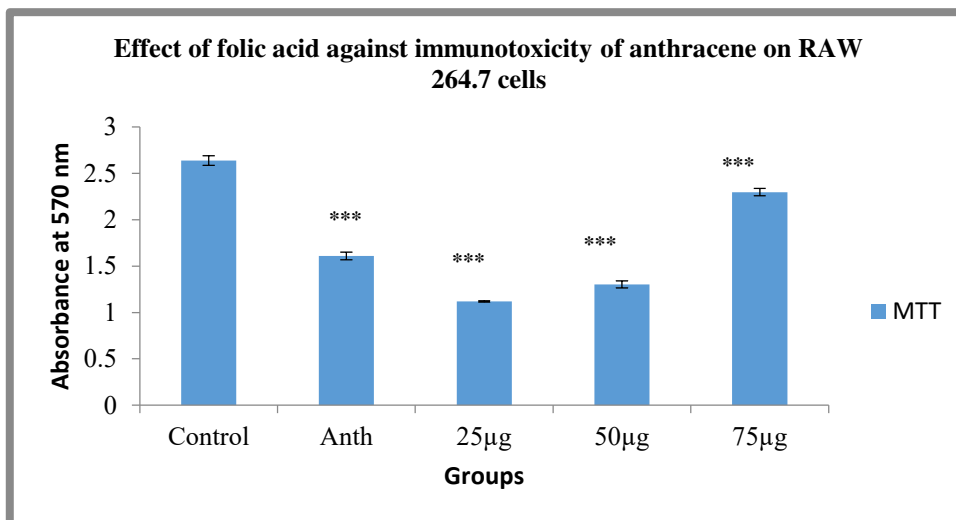


Fig 9: Protective effects of Folic acid against anthracene induced toxicity -MTT assay

Values were expressed as mean \pm SD. Statistical analysis by Students-t test. Comparisons were made between untreated control Vs treated groups. *** $p < 0.001$

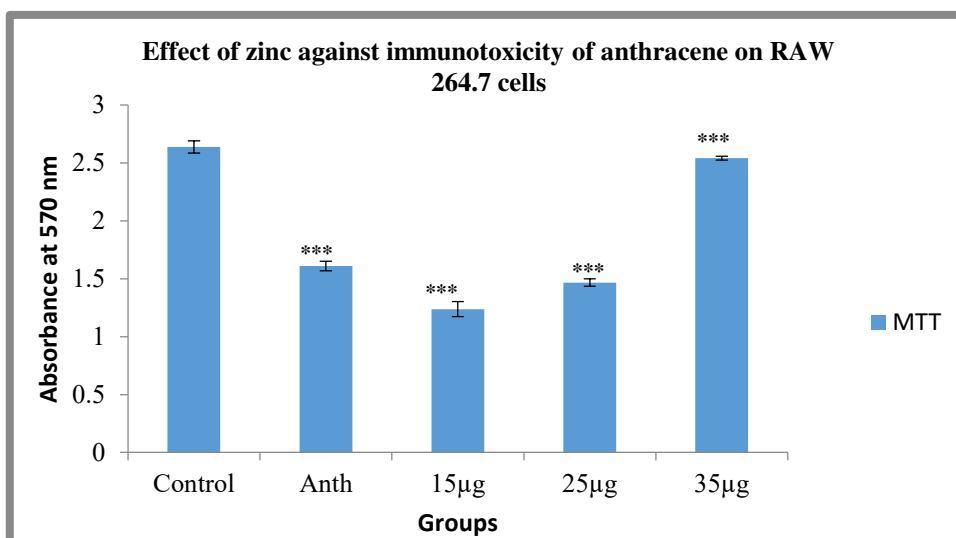


Fig 10: Protective effects of Zinc against anthracene induced toxicity- MTT assay

Values were expressed as mean \pm SD. Statistical analysis by Students-t test. Comparisons were made between untreated control Vs treated groups. *** $p < 0.001$

3.2 Effect of micronutrients on the viability of macrophage cells during immunotoxicity induced by naphthalene and anthracene.

Crystal violet (CV) cell cytotoxicity assay is one of the common methods used to detect cell viability or drug cytotoxicity. Figures 11 and 12 illustrate, the effect of micronutrient supplementation against PAH-induced immunotoxicity *in vitro*. CV is a triarylmethane dye that can bind to ribose type molecules such as DNA in nuclei. The CV staining is directly proportional to the cell biomass and can be colorimetrically measured at 570 nm. CV staining is a quick and versatile assay for screening cell viability under diverse stimulation or inhibition conditions. The precision and reliability of the crystal violet test is comparable to that of MTT assay and hence in the current study the test was performed to confirm the results of MTT assay. This test also has the advantage that simple staining of the cells help to understand the change in morphology of the cells upon treatment with a test compound by light microscopy analysis. In the current study, the results of crystal violet test were in agreement with the results of the MTT assay. Treatment with naphthalene and anthracene induced toxicity and inhibited the

proliferation of macrophages as compared to control. This decrease in cell viability and proliferation upon treatment with naphthalene and anthracene was found to be statistically significant ($P < 0.001$). Pretreatment of the cells with the micronutrients Vitamin A, Vitamin C, Folic acid and Zinc elicited appreciable protection against naphthalene and anthracene induced inhibition of cell proliferation. This is evident from the statistically significant increase in cell viability in the drug treated groups as compared to the PAH treated groups. Previous reports by Maggini et al., 2020²⁴ and Capone et al., 2019²⁵ provide support to the findings of the current study. Plate 1 and Plate 2 show the results of crystal violet staining of the cells as assessed by light microscopy analysis. In tandem with the colorimetric crystal violet test, a drastic reduction in cell numbers was observed in the cells treated with naphthalene and anthracene as compared to control ($P < 0.001$). Cells pretreated with the test compounds prior to exposure to PAH naphthalene and anthracene showed an increase in cell numbers as evident from the increased number of purple stained cells. This shows a cell proliferative response induced by the antioxidants under experimentally induced immunosuppressive conditions.

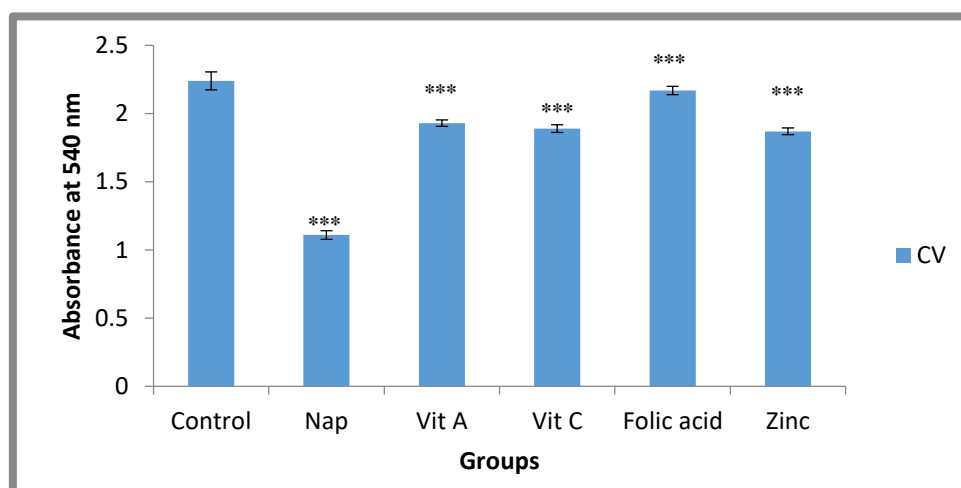


Fig 11: Effect of micronutrient supplementation against naphthalene induced toxicity -Crystal violet test

Values were expressed as mean \pm SD. Statistical analysis by Students-t test. Comparisons were made between untreated control Vs treated groups. *** $p < 0.001$

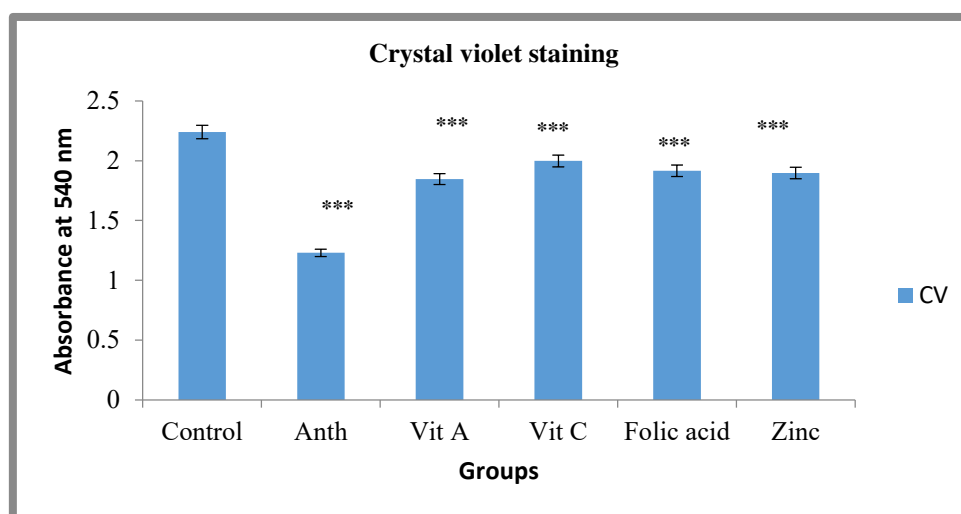
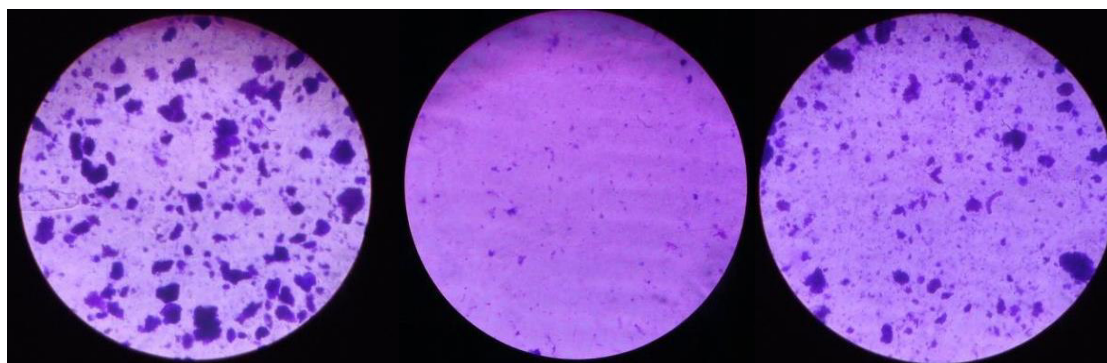


Fig 12: Effect of micronutrient supplementation against anthracene induced toxicity - Crystal violet test

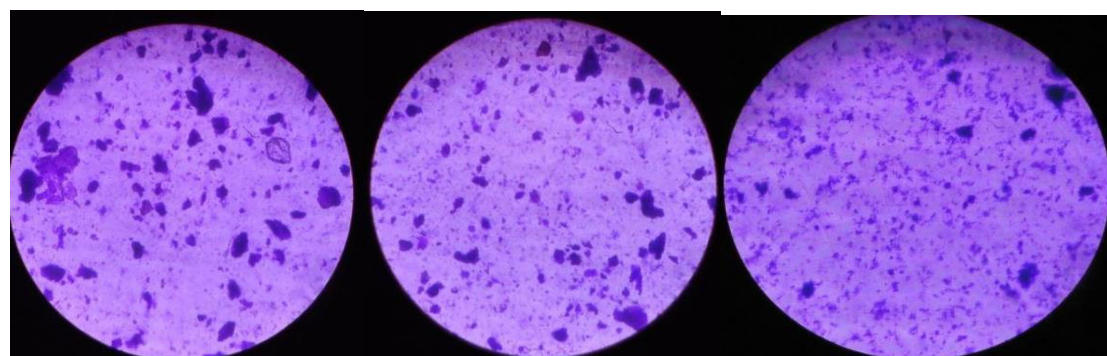
Values were expressed as mean \pm SD. Statistical analysis by Students-t test. Comparisons were made between untreated control Vs treated groups. *** $p < 0.001$



Control

Naphthalene

Naphthalene + Vitamin A

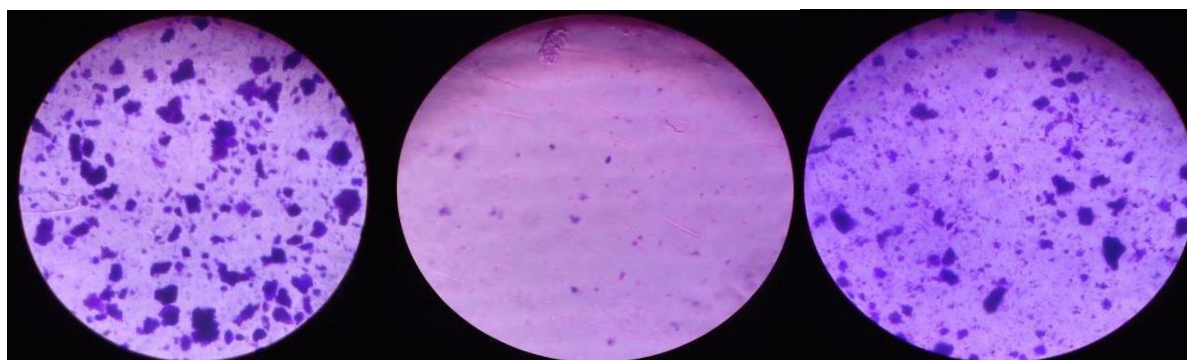


Naphthalene + Vitamin-C

Naphthalene + Folic acid

Naphthalene + Zinc

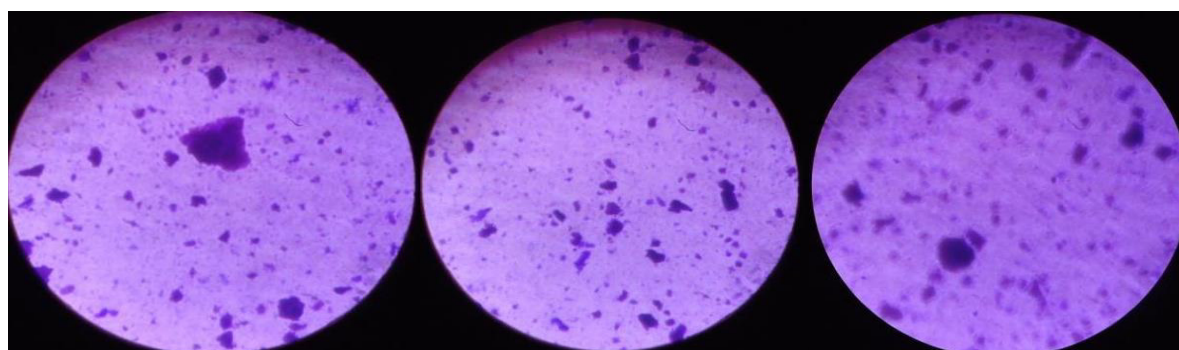
Plate 1: Effect of micronutrient supplementation against naphthalene-induced immunotoxicity- Crystal violet staining (Magnification: 40 X)



Control

Anthracene

Anthracene + Vitamin -A



Anthracene + Vitamin-B

Anthracene + Folic acid

Anthracene + Zinc

Plate 2: Effect of micronutrient supplementation against anthracene-induced immunotoxicity- Crystal violet staining (Magnification: 40 X)

3.3 Effect of micronutrients on LDH leakage from macrophage cells during immunotoxicity induced by naphthalene and anthracene.

Lactate dehydrogenase is a cytosolic enzyme which leaks into the extracellular medium when there is a compromise in the cell membrane integrity and permeability. Increased levels of LDH into the culture supernatant indicate extensive injury to the cell membrane and hence LDH assay is a reliable indicator of cytotoxic index of test compounds on the cells ²⁶. Figures 13 and 14 illustrate the effect of micronutrient supplementation against naphthalene and anthracene induced immunotoxicity on macrophages *in vitro*. Increased leakage of LDH was observed in the culture supernatant of the cells treated with naphthalene and anthracene as compared to the control cells ($P < 0.001$). Group of cells treated with the test compounds (Vitamin A, Vitamin C, Folic acid and Zinc) showed decreased leakage of LDH as compared to the naphthalene/anthracene treated cells. This indicate a preconditioning effect of the compounds on the macrophage

cells. Results of the LDH assay was in line with the results of MTT assay, crystal violet test and phase contrast microscopy analysis, wherein a cytoprotective, lymphoproliferative response was observed when the cells were pretreated with the micronutrients prior to exposure to PAH naphthalene and anthracene. Hence, the results of cell growth and viability assays indicate a growth inhibitory immunosuppressive effects of naphthalene and anthracene on the cells, whereas pretreatment with micronutrients inhibited the cytotoxicity, exerted a cell proliferative effect thereby indicating modulation of the immune system. The results from this part of the study is totally in agreement with the earlier reports that implicate the relevance of optimal nutrition containing micronutrients in shaping up the efficiency of the immune system ²⁷⁻²⁹. To confirm, whether the compounds were able to prevent the generation of Reactive Oxygen Species (ROS) and resultant induction of cell death (apoptosis), the DCFDA analysis which is a measure of reactive oxygen species generation and rhodamine 123 assay which is a measurement of mitochondrial membrane potential were performed.

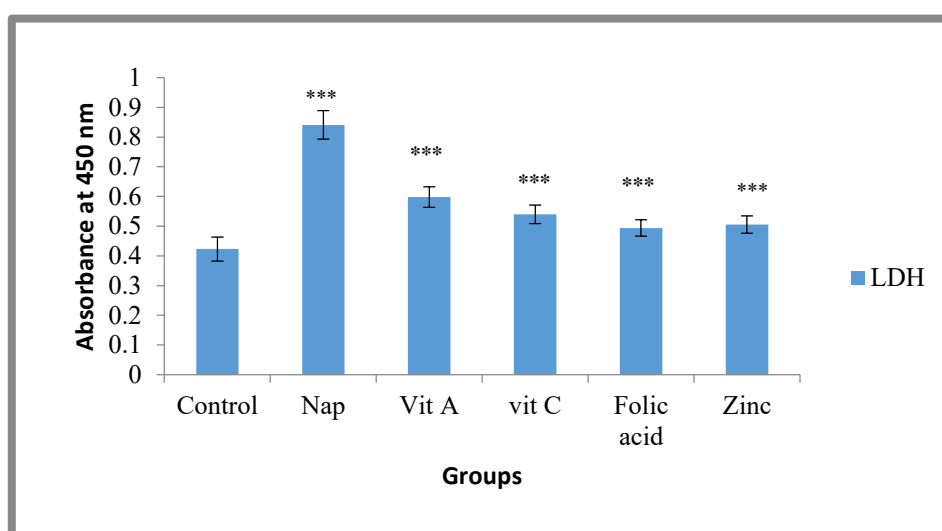


Fig 13: Effect of micronutrient supplementation against naphthalene induced toxicity – LDH leakage test

Values were expressed as mean \pm SD. Statistical analysis by Students-t test. Comparisons were made between untreated control Vs treated groups. *** $p < 0.001$

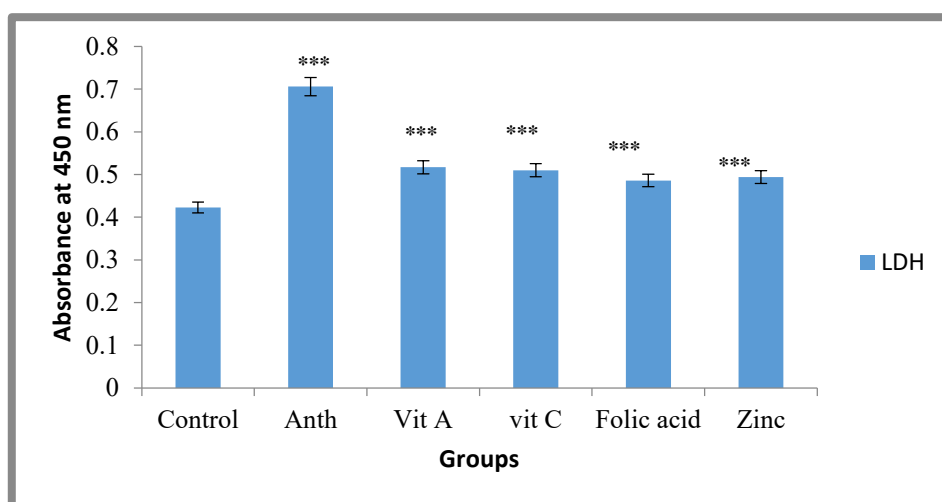


Fig 14: Effect of micronutrient supplementation against anthracene induced toxicity – LDH leakage test

Values were expressed as mean \pm SD. Statistical analysis by Students-t test. Comparisons were made between untreated control Vs treated groups. *** $p < 0.001$

3.4 Effect of micronutrients on ROS generation by macrophage cells during immunotoxicity induced by naphthalene and anthracene.

Free radicals are the presumptive markers of oxidative stress and a number of reports implicate the role of free radicals, reactive oxygen species and oxidative stress in the pathogenesis of many diseases including neurodegeneration, cardiovascular disease, diabetes and immunosuppression. Figures 15 and 16 shows the effect of micronutrient supplementation against ROS generation induced by naphthalene and anthracene as measured by DCFDA analysis. Exposure to naphthalene and anthracene resulted in a statistically significant increase ($P < 0.001$) in the generation of ROS as compared to control. This indicate the severity of oxidative stress induced as a result of exposure to poly aromatic hydrocarbons. Treatment with the micronutrients were able to prevent the induction of oxidative stress inflicted by naphthalene and anthracene which was evident from the decreased levels of ROS in the group of cells treated with Vitamin A, Vitamin C, Folic acid and Zinc respectively. All the four micronutrients under examination, showed a significant decrease in the generation of ROS as compared to the group of cells exposed to naphthalene and anthracene. This implicate that all the three compounds are effective in scavenging the

free radicals and relieve the oxidative stress induced by the PAHs. All the four micronutrients used in the study were potent antioxidants and hence the observed decrease in ROS production upon micronutrient treatment could be attributed to the strong immunopotentiating antioxidant effects of these compounds³⁰.

3.5 Effect of micronutrients on mitochondrial membrane permeability of macrophage cells during immunotoxicity induced by naphthalene and anthracene.

Cellular health closely correlates with active mitochondrial function and hence measuring mitochondrial membrane potential (MMP) could be a reliable and sensitive indicator of cell death caused by stress, toxic insults and other pathological conditions. Fluorescent Cationic dyes are frequently employed tools for the determination of MMP accurately. The mitochondrial membrane potential ($\Delta \Psi_M$) is the major component of the proton motive force (Δp)^{31,32}, which is in turn the central intermediate of aerobic energy production, and driving force of other physiological processes in mitochondria, such as Ca^{2+} uptake, antioxidant defence (NADPH generation at the transhydrogenase) or heat production of brown fat.

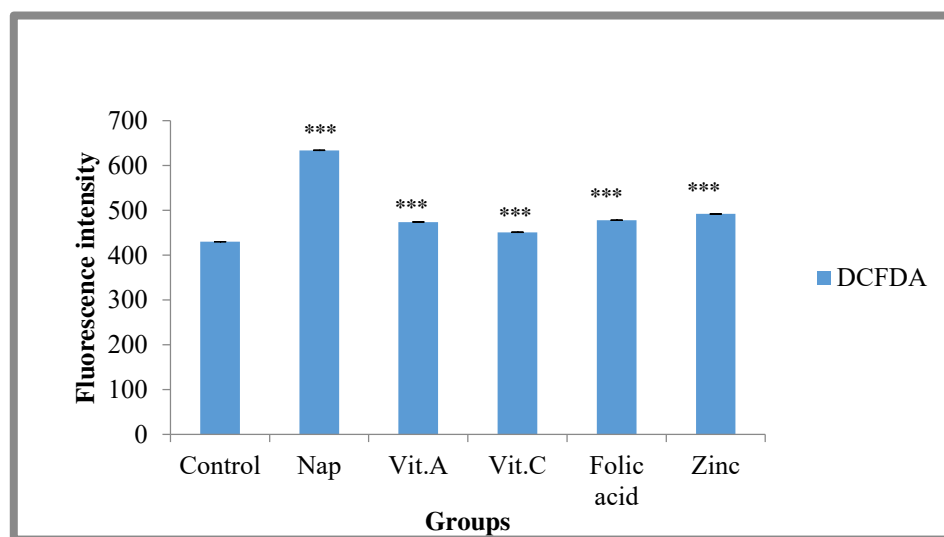


Fig 15: Effect of micronutrient supplementation against naphthalene-induced ROS generation- DCFDA analysis

Values were expressed as mean \pm SD. Statistical analysis by Students-t test. Comparisons were made between untreated control Vs treated groups. *** $p < 0.001$

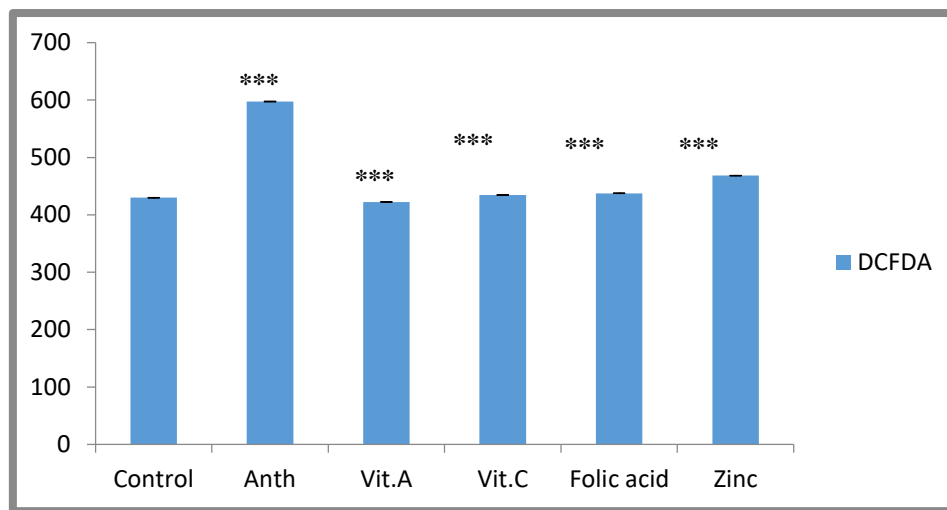


Fig 16: Effect of micronutrient supplementation against anthracene-induced ROS generation- DCFDA analysis

Values were expressed as mean \pm SD. Statistical analysis by Students-t test. Comparisons were made between untreated control Vs treated groups. *** $p < 0.001$

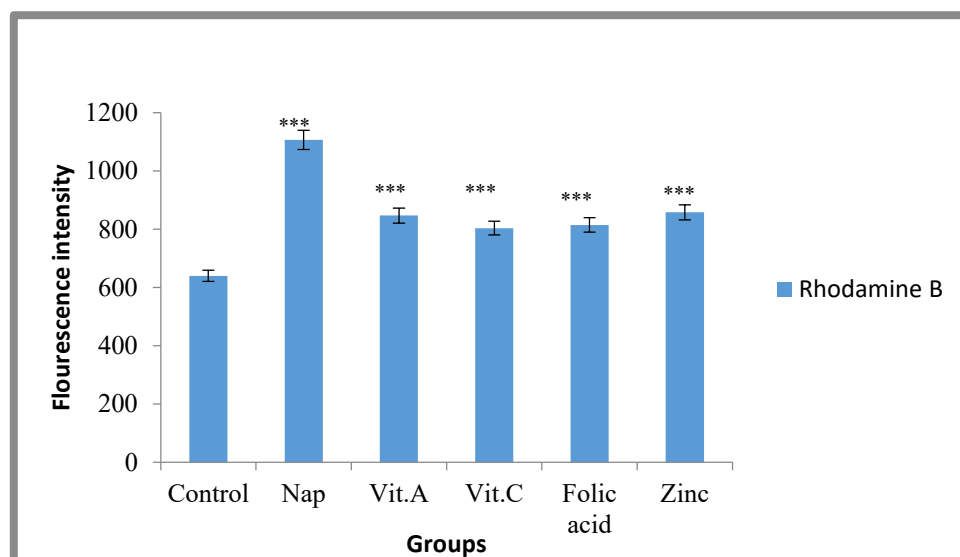


Fig 17: Effect of micronutrient supplementation against naphthalene-induced alterations in mitochondrial membrane potential- Rhodamine B analysis

Values were expressed as mean \pm SD. Statistical analysis by Students-t test. Comparisons were made between untreated control Vs treated groups. *** $p < 0.001$

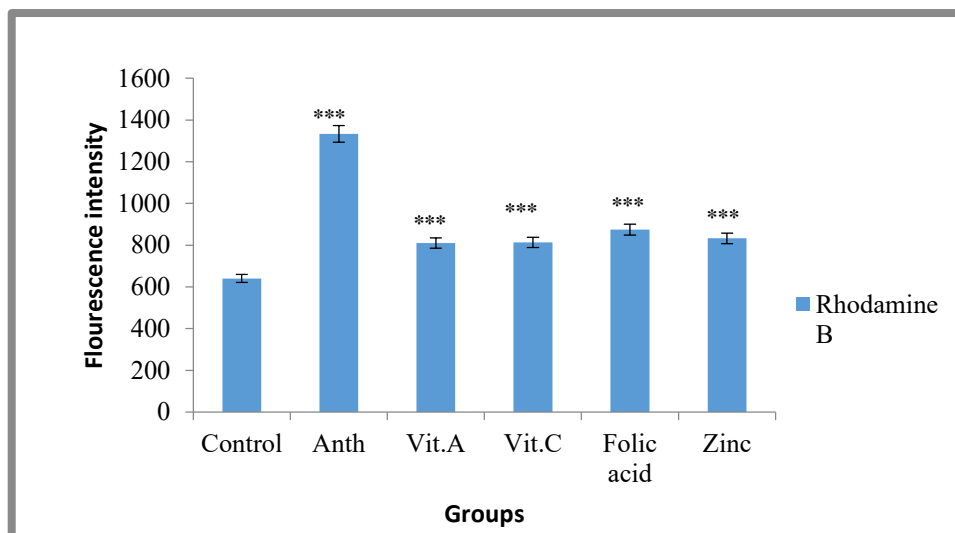


Fig 18: Effect of micronutrient supplementation against anthracene-induced alterations in mitochondrial membrane potential- Rhodamine B analysis

Values were expressed as mean \pm SD. Statistical analysis by Students-t test. Comparisons were made between untreated control Vs treated groups. *** $p < 0.001$

Rhodamine 123 (RH-123) was frequently used to monitor the membrane potential of mitochondria. Fluorescence of this dye is quenched by active mitochondrial energization and hence the mitochondrial membrane potential is directly proportional to the rate of fluorescence decay. Figure 17 and 18 shows the effect of antioxidant supplementation on alterations in the mitochondrial membrane potential during PAH induced immunotoxicity *in vitro*. Exposure to naphthalene and anthracene induced significant alterations ($P < 0.001$) in mitochondrial membrane potential wherein treatment with the Vitamin A, Vitamin C, Folic acid and Zinc could prevent these changes thereby implicating their protective effects in combating the toxicity of the PAHs. Hence, based on the preliminary assays carried out in the study, it is understood that exposure to naphthalene and anthracene inhibited macrophage cell proliferation, induced the production of free radicals, reactive oxygen species and hence severe oxidative stress resulting in immunosuppressive conditions. Pretreatment with micronutrients Vitamin A, Vitamin C, Folic acid and Zinc helped in negating the oxidative stress and mitigating the damage induced by naphthalene and anthracene and thereby alleviated the immunotoxicity induced by these compounds. All the protective effects observed are in concurrence with previous reported beneficial effects of micronutrients on human immune health³³. Further detailed studies are required to have a thorough understanding of the actual mechanisms involved behind the immunomodulatory effects of these compounds.

4. CONCLUSION

Exposure of macrophage cells to poly aromatic hydrocarbons naphthalene and anthracene resulted in immunosuppressive conditions, ROS generation and induction of cell death.

Pretreatment with micronutrients Vitamin A, Vitamin C, Zinc and Folic acid were able to prevent these changes thereby implicating a protective effect. Anthracene was found to be a more potent immunosuppressant than naphthalene. Vitamin A was found to be more effective in alleviating the immunological stress induced by the poly aromatic hydrocarbons naphthalene and anthracene. Supplementation of this vitamin or a combination of all the micronutrients used in the study together, could be beneficial to prevent the damage in high risk population who are exposed to PAH frequently due to occupational hazards.

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6. AUTHORS CONTRIBUTION STATEMENT

Dr.M. Sreepriya, corresponding author of the manuscript developed the concept, designed the work plan, helped in culture and subculture of macrophage cells, interpreted the results and arrived at conclusions. Vijay Joshi and Sudha were involved in MTT assay, LDH release assay, DCFDA and Rhodamine B assay. Muthukumaran carried out crystal violet test.

7. CONFLICT OF INTEREST

Conflict of interest declared none.

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