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In silico and in vitro testing Ascorbic Acid Protects Nicotine-Treated Human Erythrocytes

Running title: *In vitro and in silico* testing of Ascorbic Acid

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ABSTRACT

Objectives: Nicotine is a naturally occurring drug that is highly addictive and is commonly found in tobacco products. Tobacco products are one of the leading causes of lung and oral cancer worldwide. Because blood is the primary organ in contact with nicotine, our goal in this study was to confirm its effect on blood. We also investigated the protective role of ascorbic acid on nicotine toxicity.

Methods: Various blood toxicity studies were carried out using in-vitro and in-silico methods. Nicotine-induced haematological perturbation resulted in haemolysis, decreased superoxide dismutase and catalase activities, and decreased total antioxidant capacity in erythrocytes.

Results and Conclusions: The results showed that nicotine has a negative effect on red blood cells in the lysis assay and causes clots to form in nicotine-treated samples in the blood clotting analysis. Furthermore, the in-Silico method validated the in-vitro results. According to our findings, ascorbic acid has blood-protective properties. Ascorbic acid was discovered to increase SOD, catalase, and total antioxidant activity. Ascorbic acid also reduced the damage caused by nicotine to RBCs in the lysis assay and demonstrated a high level of protection against the formation of clots when the samples were treated with nicotine. The findings suggest that ascorbic acid, an antioxidant, can protect against nicotine-induced haematological damage.

Keywords : Nicotine, *In-Silico*, *In-vitro*, Ascorbic acid, Erythrocytes, antioxidant

I. INTRODUCTION

Nicotine is considered as a natural toxic substance found in the tobacco products. Nicotine is a very addictive drug, and according to a 1988 report on addiction, it is compared to heroin addiction. Nicotine is a hazardous neurotoxin that is extremely deadly and poisonous when consumed [1]. Liquid nicotine, a component of the popular e-cigarette, has recently been linked to an upsurge in poisonings. Because of

their tiny size, children are more susceptible to poisoning. When nicotine enters the body, it causes the adrenal glands to generate adrenaline, which stimulates the body and raises the blood pressure and heart rate. Nicotine is also linked with the oral and lung cancer, in the United States, 90% of lung cancer is considered to be associated with smoking. It causes narrowing of the arteries and increases blood flow to the heart and high concentration of nicotine can causes hardening of the arterial walls, which increases the risk of a heart attack. Nicotine shows adverse effect blood vessels as well. It causes blood vessels constriction and decreases in elasticity, restricting blood flow to organs. The more blood vessels constrict the stiffer and less elastic they become. Nicotine remains in the bloodstream for at least three days after smoking [2]. Understanding the effects of nicotine on placental perfusion and development is critical for reducing and preventing its negative effects. Nicotine raises the number of lymphocytes, leukocytes, and hemoglobin in the blood. It causes increase in hemoglobin distribution curve, making hemoglobin levels less useful, which leads to the anemia and reduced iron intake. NAs nicotine raises the adrenaline that can cause the blood clotting in blood vessels nicotine raises the number of RBCs significantly. Therefore, nicotine raises levels of hormones like adrenaline in the body, which can lead to an increase in blood clot formation and can cause enlarged aorta, and atherosclerosis, forming plaque on the artery wall [3,17]

High concentration of nicotine in the body causes an antioxidant imbalance. This imbalance leads to harmful effects on body tissues and organs [4]. The antioxidant defence system of the body has both enzymatic and non-enzymatic antioxidants. Glutathione is an important antioxidant whose function is in xenobiotic metabolisms, such as detoxification, which helps in the liver's detoxification process. Antioxidant enzymes such as catalase, peroxidase, and glutathione reduce the free radicals by in the cells. According to Fagerstrom 2014, antioxidants protect the blood against the nicotine poisoning and including vitamins E and C, as well as carotenoids, may help protect the red blood cells from free radical damage [1]. Mongi et.al;2011 state that ascorbic acid plays a vital role in protecting blood from free radical related toxicity. Increased quantity of ascorbic acid intake can elevate the antioxidant level up to 30% which can result in additional protection from free radical related damage to red blood cells. In the present study authors tried to find out the effect of nicotine toxicity on the red blood cells and the role of ascorbic acid in protecting or reducing nicotine related risk [5].

II. METHODS

Reagents

We bought tocopherol, ascorbic acid, and pure nicotine. Sisco Research Laboratories Pvt. Ltd. produced dimethyl sulphoxide (DMSO) (SRL). From Panreac Quimica, NaCl, Na2PO4, 30% H2O2, and NaH2PO4 were extracted. Different antioxidants and anticancers were diluted by adding enough substances to DMSO to dissolve them. The phosphate-buffer saline was made using NaCl, Na2HPO4, and NaH2PO4 in autoclaved double distilled water. An assay kit for superoxide dismutase (SOD) was purchased from Bangalore.

Blood Sampling

Healthy volunteers donated the 5ml blood with informed consent (All the volunteers were of age group 20-35 years). The blood samples were collected in heparin coated tubes. All the experiments were performed in triplicates and mean value was taken for the final calculations.

Osmotic Fragility Assay

Osmotic fragility assay or RBC lysis assay was conducted to study the nicotine related osmotic haemolysis [6]. Briefly, 1ml of heparinised blood sample was incubated for half an hour at 37°C in absence of any test chemical (Control), in presence of 1µg/ml concentration of nicotine, in presence of 1µg/ml ascorbic acid and in presence of both ascorbic

acid and nicotine in separate tubes. PBS was diluted in distilled water with concentrations ranging from 10 to 1 g/L. the treated blood was then added to the different concentrations of PBS (with decreasing concentrations) The solution was then slowly mixed by tapping the tubes. All the samples were then incubated for 30 minutes. All the tubes were centrifuged at 3000 rpm for 5 minutes. The supernatant was collected and the OD at 540nm was recorded. The OD of the collected solutions was indicating the degree of RBCs lysis [7]. The lysis percentage was calculated by dividing the OD of the supernatant obtained from a particular saline concentration by the OD of the standard (1g/L) representing 100% haemolysis. The lysis percentage was plotted against the concentration of saline solutions to create osmotic fragility curves. The curve yielded the MEF25, MEF50, and MEF75 (mean erythrocyte fragility) values, which are the saline concentrations at which 25%, 50%, and 75% of red blood cells haemolyzed (at standard pH and temperature).

Treatment Conditions

Two different conditions were used to study the effect of nicotine and ascorbic acid together and separate tubes, the pre-treatment condition where the ascorbic acid was added to the blood 30 minutes earlier before adding the nicotine in the same sample. In the second condition both the chemicals were added together at the same time (Co-treatment).

Statistical Analysis

The results were recorded as SME. $P < 0.05$ was considered statistically significant.

Preparation of Erythrocyte Lysate

For erythrocyte lysate preparation, the erythrocytes were washed four times with 0.9% NaCl, and then the final volume was mentioned up to 2.0 ml by using old sterilized water. The final 25- fold dilution prepared for the final use.

Superoxide Dismutase Assay

The enzyme superoxide dismutase (SOD) activity was carried out using has been investigated by using superoxide dismutase assay kit (Biodiagnostic)[8]. Briefly, the 500 µl /per sample lysate was treated with nicotine, ascorbic acid, and nicotine + ascorbic acid for 30 minutes. All the reagents were prepared as per instruction then nitroblue tetrazolium solution (NBT) and NADH (1 ml each) were mixed together immediately before use. 1000 times diluted Phenazine methosulphate (PMS) was prepared prior to use. 100µl of treated lysate was collected and mixed with the working reagent and PMS (100 µl). The samples were analysed by recording the OD at 560 nm for 5 minutes.

Catalase Assay

Catalase assay was conducted in erythrocyte lysates[9,10]. In brief the 100ul treated and untreated samples were transferred in the quartz cuvette which were containing the 2.90 mL H_2O_2 solution (0.036% in 50 mM potassium buffer). The OD was taken for 180 seconds, Bbuffer was used as blank.

***In-vitro* Lysis assay**

Four blood samples were prepared with different concentrations of ascorbic acid and nicotine as follows; Control sample, treated with nicotine, treated with ascorbic acid, and the combination of ascorbic acid and nicotine. All the samples were prepared in triplicates and then incubated for 30 minutes at room temperature. After the incubation the 10 ul of each sample was placed on glass slide then stained with the Methylene Blue. The slides were placed under the microscope and observed for the lysis of RBC [11].

***In-vitro* Blood clotting Analysis**

Four blood samples were collected from the volunteers in heparin treated blood collection tubes (A control sample, a nicotine-treated sample, an ascorbic acid-treated sample, and a nicotine-and-ascorbic acid-treated sample) were all incubated for 30 minutes. After that, the samples were placed on slides and dried at room temperature. Then all the slides were observed under microscope at 100X.

Plasma preparation

The blood was centrifuged for 10 minutes at 3000 rpm. At 4 degrees Celsius without disturbing the white buffy layer, the top yellow plasma layer was collected.

Total Antioxidant Capacity Assay

The antioxidative capacity is determined by reacting antioxidants in the sample with a predetermined amount of exogenously provided hydrogen peroxide. Antioxidants in the sample remove a portion of the provided hydrogen peroxide. Colorimetric determination of residual H_2O_2 is accomplished through an enzymatic reaction that involves the conversion of 3,5, dichloro –2– hydroxy benzensulphonate to a colored product [12]. The determination of the antioxidative capacity is performed by using total antioxidant capacity assay kit available commercially. 500 µl of plasma was treated with nicotine, ascorbic acid, and nicotine with ascorbic acid in separate microcentrifuge tubes for 30 minutes. The working reagent was prepared by adding 1.5 ml of Chromogen and 1.5 ml Enzyme – Buffer immediately before use. The H_2O_2 was diluted 1000 times prior to use. After mixing 20µl of treated plasma with 500 µl of substrate (H_2O_2) and incubating for 10 minutes at 37°C, 500 µl of working reagent was added to the tube. After 5 min Absorption was measured at five hundred nanometers.

***In Silico* analysis using HEX8.0**

In the current research, bioinformatics tools such as PDB (Protein Data Bank), and software such as Hex 8.0 and Chem draw were used. Chem draw is a professional tool used mostly by researchers to design and communicate chemical structures. It also aids in the drawing of chemical molecules. The structure of nicotine was drawn using chem-draw software [13]. Hex8.0 was used for docking. The protein structures were obtained from Protein Data Bank (PDB) which provides the details about various proteins obtained through X-ray crystallography, NMR, and other techniques [14].

III.RESULTS AND DISCUSSION

The mean erythrocyte fragility was used to summarize all of the osmotic fragility assay results (MEF). The MEF levels didn't significantly alter after the pre-treatment (**Table-2)** However, when both chemicals were applied simultaneously; the co-treatment findings amply demonstrate the protective role of ascorbic acid against nicotine-induced osmotic lysis of red blood cells (**Figure-2 & 3**). Results from a superoxide dismutase (SOD) study showed that nicotine greatly reduced the erythrocytes' SOD activity, but that this harmful effect on SOD was significantly reduced when combined with ascorbic acid **(Figure-4).** Results of the catalase experiment showed that ascorbic acid neutralized the toxic effects of nicotine on the catalase enzyme **(Figure-5).** Total antioxidant capacity of human erythrocytes that had been exposed to nicotine, ascorbic acid, or both. The findings show that nicotine has a negative impact on red blood cells' overall antioxidant activity, but that ascorbic acid, when combined with nicotine, has a protective effect **(Figure-6)**. Ascorbic acid significantly protected against nicotine-related toxicity, according to an in-vitro lysis assay **(Figure-7).** The effects of nicotine on the clotting of blood treated with heparin were also examined in blood samples, and the results showed that the presence of ascorbic acid in nicotine-treated cells inhibits the clotting of red blood cells **(Figure-8).** The docking results are presented in Figure 9, which enables the receptor molecule to rotate on the Z axis by docking nicotine with various proteins and the Hex program. 16 From the acquired data, nicotine demonstrated a substantial E-total value with superoxide dismutase and heparin **(Figure-10)**

Discussion

Nicotine is one of the most addictive naturally occurring drugs commonly found in the tobacco products. The tobacco products are one of the most common causes of lung and oral cancer all over the world. The effects of nicotine on the body of a human

being are varied .Our study proves the toxic effects of nicotine on human blood as it was causing the oxidative damage to the blood cells. Our data demonstrated that nicotine induces oxidative stress in the human erythrocytes, osmotic lysis, and reduction in the major antioxidant enzymes SOD and catalase. And reduced total antioxidant activity. By completing two experiments designed by us in the laboratory, lysis assay and clotting analysis we were able to demonstrate the severity of nicotine's damage to RBCs, and the clotting formation. According to our results, treating nicotine with ascorbic acid reduced nicotine adverse effects. Our research shows that ascorbic acid has protective effects on human blood. The results of the osmotic fragility assay indicated that when the cells were treated with nicotine, the erythrocyte damage caused by osmotic stress was high, but when the cells were treated with nicotine in the presence of ascorbic acid the effect was decreased. It was found that ascorbic acid increase SOD activity in the superoxide dismutase assay. Nicotine inhibited catalase activity, whereas ascorbic acid increased it. Ascorbic acid demonstrated promising results in terms of increasing total antioxidant activity. Ascorbic acid also reduced the damage caused by nicotine to RBCs in the lysis assay and it showed a high protection from the formation of clots that formed when treating the samples with nicotine, as we saw in the previous results [15]. The *in-silico* data validate the result of in-vitro results. The results indicate that all the selected proteins have very good binding affinity to nicotine, especially Heparin [16].

IV. CONCLUSION

Nicotine is considered to be a very toxic naturally occurring natural product. Nicotine toxicity occurs when nicotine starts to exhibit toxic effects on an individual. Nicotine also has an adverse effect on haematological parameters. Our research shows that nicotine causes acute haemolysis in erythrocytes. Nicotine reduces the activity of SOD and catalase as well as the total antioxidant capacity. Using in-vitro and in-silico methods in our research the results indicate the damaging effect of nicotine can be minimized by treating nicotine with ascorbic acid.

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FIGURE CAPTIONS:

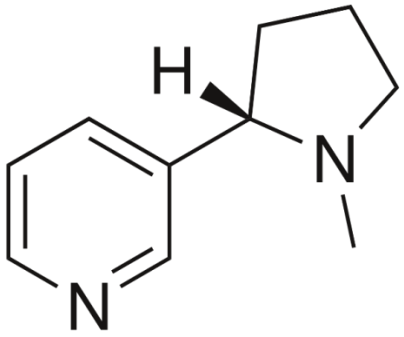

Figure 1. Nicotine structure (Prepared on Chemdraw)

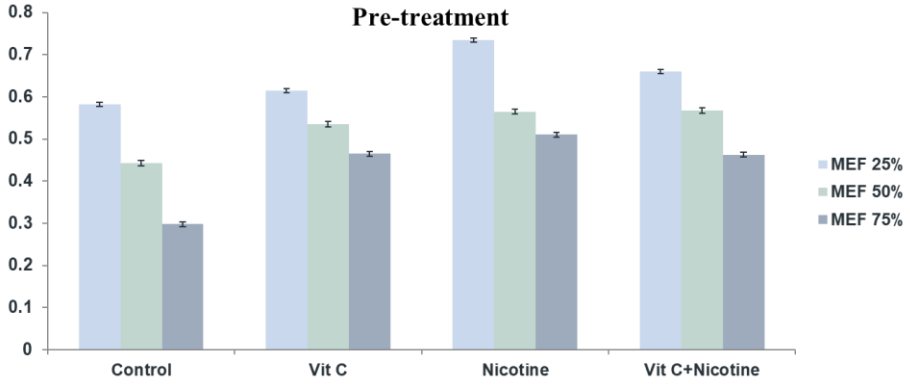

Figure 2 Pre-treatment of ascorbic acid with Nicotine resulting in mean erythrocyte fragility (MEF). The results indicate that the pre-treatment of red blood cells with ascorbic acid protects the osmotic damage caused by nicotine.

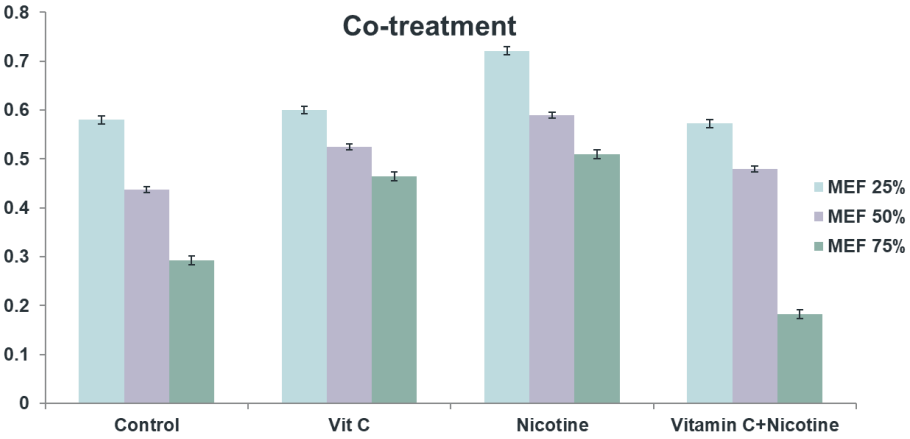

Figure 3 Co-treatment of ascorbic acid with nicotine resulting in mean erythrocyte fragility (MEF). The results indicate that the damaging effect of nicotine can be reduced by treatment of the red blood cells with ascorbic acid along with nicotine.

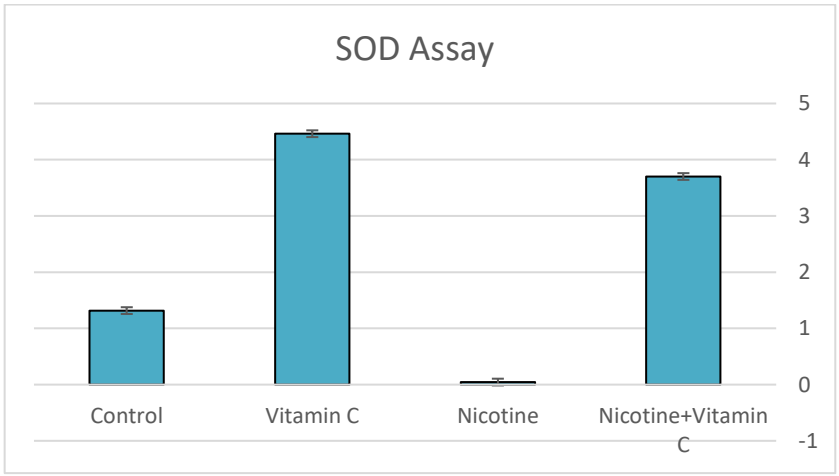

Figure4. Mean superoxide dismutase (SOD) activity (units/ml) of erythrocytes in nicotine treated, and ascorbic acid treated, nicotine with ascorbic acid-treated human erythrocyte.

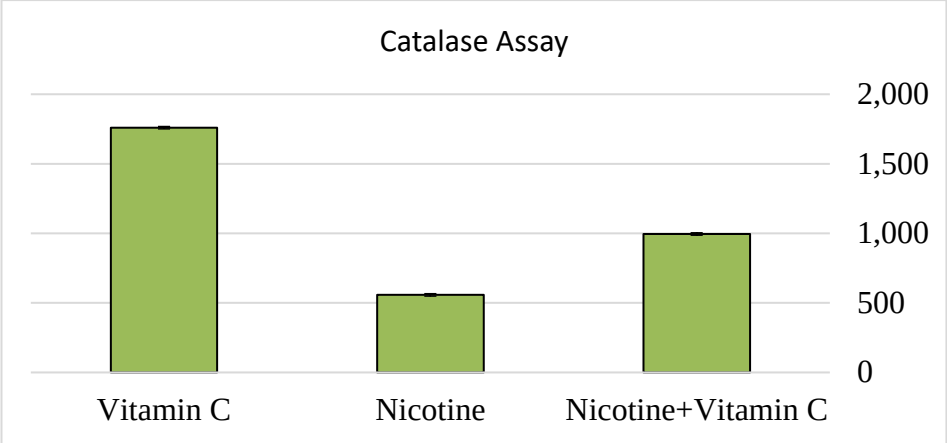

Figure5. Catalase activity in nicotine treated, ascorbic acid treated, nicotine with ascorbic acid-treated human erythrocyte.

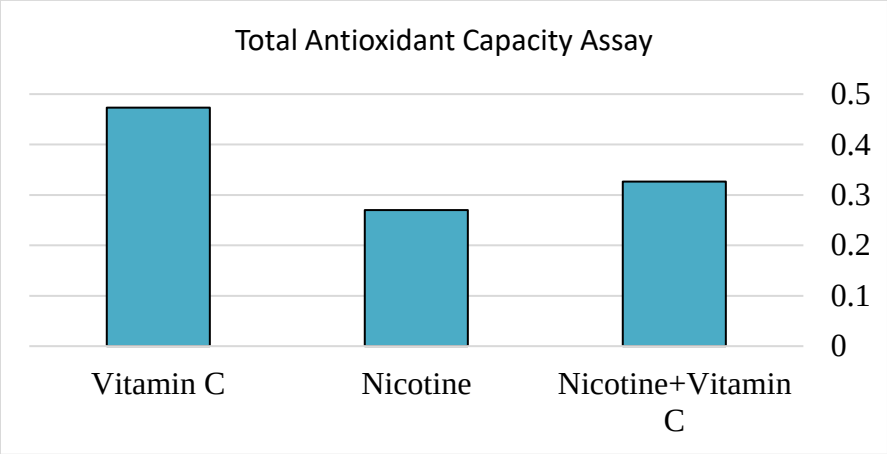

Figure6. Total Antioxidant Capacity in nicotine treated, ascorbic acid treated, nicotine with ascorbic acid-treated human erythrocyte.

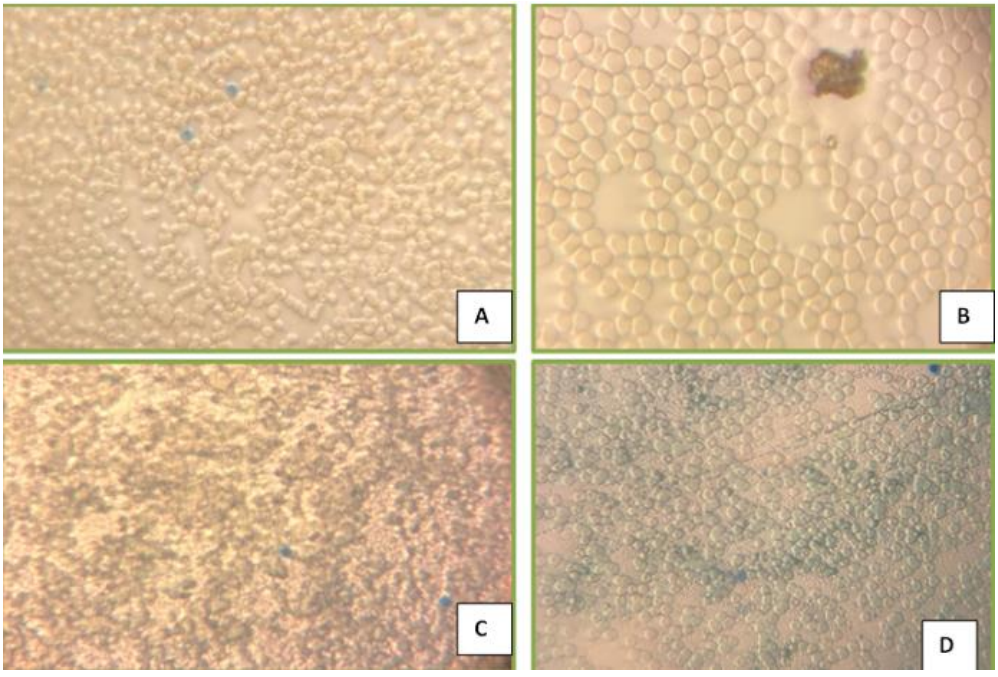

Figure 7: In-vitro Lysis assay A) Control (B) Ascorbic acid treated (C) Nicotine treated (D) Nicotine+ Ascorbic Acid treated

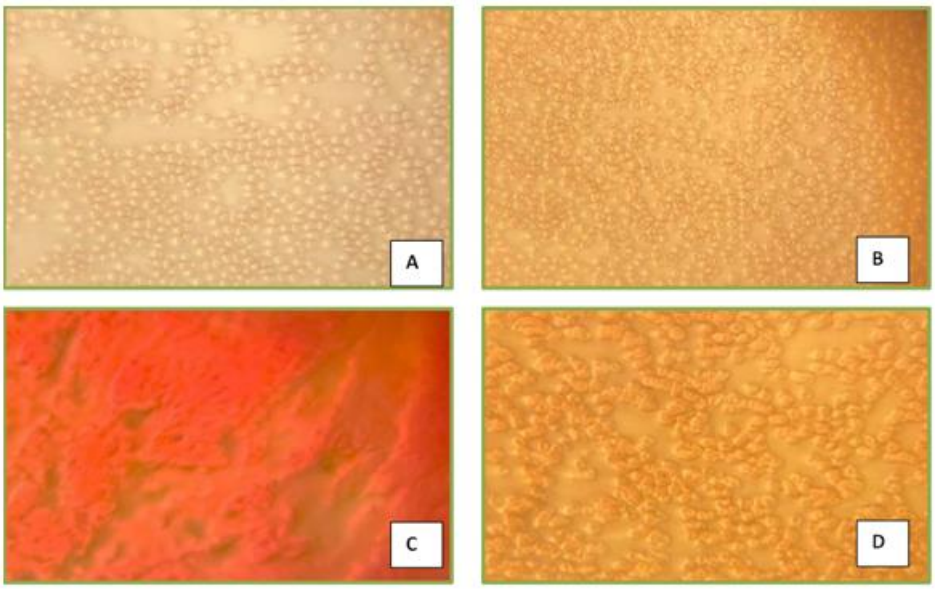

Figure 8: *In-vitro* Blood clotting Analysis (A) Control (B) Ascorbic acid treated (C) Nicotine treated (D) Nicotine+ Ascorbic Acid treated

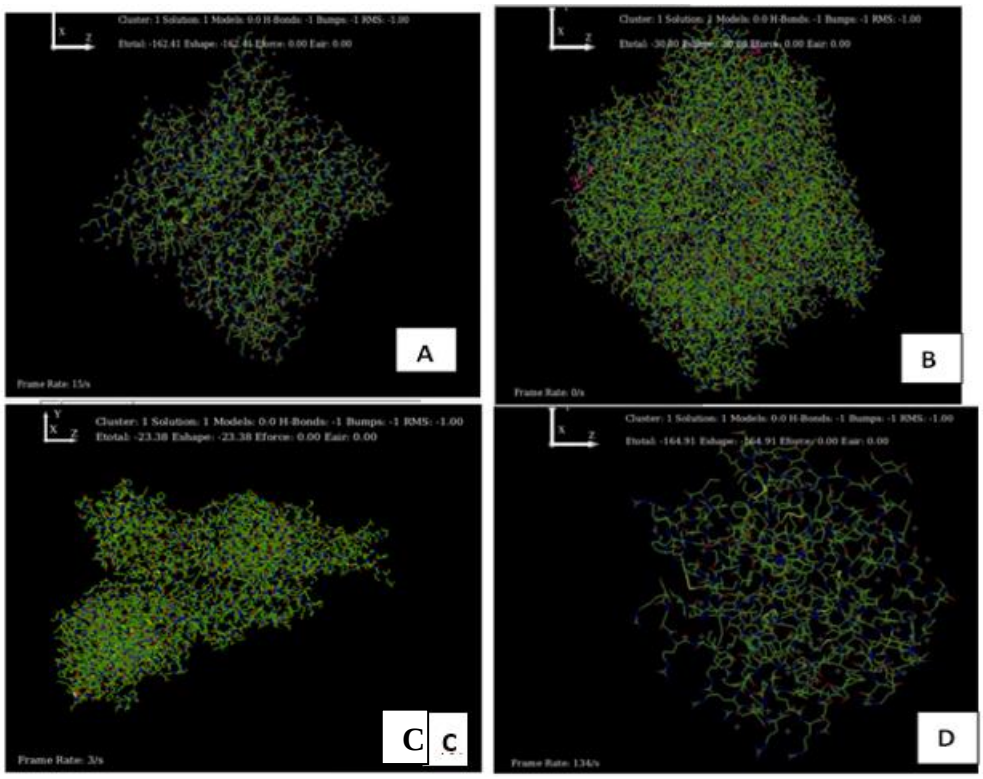

Figure9: Docking between the nicotine with different proteins (A).Superoxide dismutase, (B) Catalase, (C) Fibrinogen, (D) Heparin

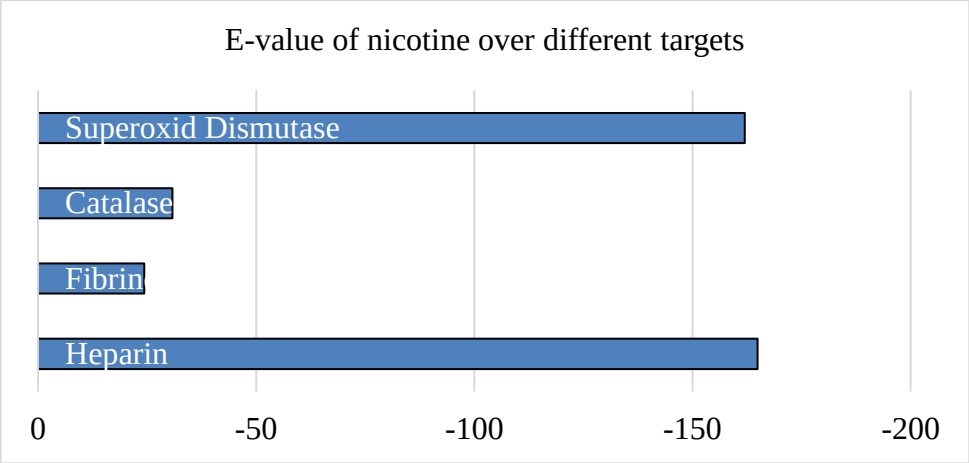

Figure10: E-value of nicotine over different Protein targets A).Superoxide dismutase, (B) Catalase, (C) Fibrinogen, (D) Heparin

TABLES

Parameters	Range
Correlation type	Shape Only
FTT mode	3D Fast lite
Grid Dimension	0.6
Receptor Range	180
Ligand Range	- 180
Twist Range	- 360
Distance Range	- 40

Table1. The parameters used in the docking process

SNo.		MEF25%	MEF50%	MEF75%
1	Control	0.5825±0.08536	0.4425±0.05146	0.2975±0.09146
2	Ascorbic Acid	0.615±0.014142	0.535±0.012583	0.465±0.0125
3	Nicotine	0.735±0.005774	0.565±0.017321	0.51±0.034641
4	Ascorbic Acid +Nicotine	0.66±0.014142	0.5675±0.012583	0.4625±0.012583

Table 2. Pre-treatment of ascorbic acid resulting in mean erythrocyte fragility (MEF)

SNo.		MEF 25%	MEF 50%	MEF 75%
1	Control	0.58±0.052	0.4375±0.996	0.2925±0.996
2	Ascorbic Acid	0.6±0.471405	0.525±0.005774	0.465±0.005774
3	Nicotine	0.721±0.0256	0.59±0.0266	0.51±0.522
4	Ascorbic Acid +Nicotine	0.5725±0.052	0.48±0.066	0.1825±0.002

Table 3 Co-treatment of ascorbic acid resulting in mean erythrocyte fragility (MEF).

Ethical approval: No ethical approval was needed as the blood samples were obtained from healthy volunteers with their consents.

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Conflict of Interest: The authors declare that there is no conflict of interest

V. REFERENCES

[1]. Fagerstrom, K. Nicotine: Pharmacology, toxicity, and therapeutic use. Journal of Smoking Cessation, 2014; 9(2), 53–59.

[2]. Mishara,A, et al. . Harmful effects of nicotine. Indian J Med Paediatr Oncol, 2015;36(1), 24–31.

[3]. Thompson, J., et al. Development of screening assays for nanoparticle toxicity assessment in human blood: preliminary studies with charged Au nanoparticles. Nanomedicine, 2012; 7(9), 1355–1364.

[4]. Uchida S and Kagitani F. Effects of nicotine on regional blood flow in the olfactory bulb in response to olfactory nerve stimulation. The Journal of Physio. Sci., 2020; 70(1), pp.1-10.

[5]. Mongi S. et al. Protective effects of vitamin C against haematological andbiochemical toxicity induced by deltamethrin in male Wistar rats. Ecotoxicol Environ Saf, 2011; 74(6), 1765-1769.

[6]. Jacobs MH. Osmotic properties of the erythrocyte. III. The applicability of osmoticlaws to the rate of hemolysis in hypotonic solutions of non-electrolytes. Biol Bull,1932; 62(2), 178–194.

[7]. Li YQ, Hu R, Zhong LH, et al. Synergistic effect of trehalose and saccharose pretreatment on maintenance of lyophilized human red blood cell quality. Trop J Pharm Res 2016; 15(3):527–533.

[8]. Nishikimi M, Rao NA and Yagi K. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochem BiophysRes Commun, 1972; 46(2): 849–854.

[9]. Aebi H.Catalase. Meth Enzymol 1984; 105: 121–126.

[10]. Aryal S. (2021). Catalase test- principle, uses, procedure, result interpretation with precautions. Retrieved from https://microbiologyinfo.com/catalase-test-principleuses-procedure-result-interpretation-with-precautions/

[11]. Islam, M., Aryasomayajula, A. and Selvaganapathy, P. A Review on Macroscale and Microscale Cell Lysis Methods, Micromachines , 2017;8(3), 83-90.

[12]. Koracevic, D, Koracevic. G. et al, Total Antioxidant Capacity. J Clin Pathol, 2001;54, 356-361

[13]. Li YQ, Hu R, Zhong LH, et al. Synergistic effect of trehalose and saccharose pretreatment on maintenance of lyophilized human red blood cell quality. Trop J Pharm Res 2016; 15(3):527–533.

[14]. Berman HM. The Protein Data Bank: a historical perspective. Acta Crystal Section A: Foundations of Crystallography 2008; 64(1), 88-95.

[15]. Bates S and Weitz, J. Coagulation assays. Circulation, 2005; 112(4), 53-60.

[16]. Vishnuvarthan VJ, Lakshmi KS & Srividya AR. In-silico screening of flavonoids targeted for death receptors in cancer by using Hex molecular docking. Journal of Young Pharmacists, 2017; 9(2), 168.

[17]. Lo, J. O., Schabel, M. C., Roberts, V. H., Morgan, T. K., Rasanen, J. P., Kroenke, C. D., Shoemaker, S. R., Spindel, E. R., & Frias, A. E.. Vitamin C supplementation ameliorates the adverse effects of nicotine on placental hemodynamics and histology in nonhuman primates. Am. J. Obstet. Gynecol,; 2015 :212(3)

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