



Effect of Oxidative Phytochemicals on Nicotine-stressed UMNSAH/DF-1 Cell Line

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ABSTRACT

Nicotine is a parasympathomimetic alkaloid found in the nightshade family of plants (Solanaceae) and is a cholinergic drug. It acts directly by stimulating the nicotinic or muscarinic receptors or indirectly by inhibiting cholinesterase, promoting acetylcholine release, or by other mechanisms. 3% of tobacco or one cigarette yields 1 mg of nicotine. As nicotine enters the body, it disturbs the healthy functioning of the body. In this study, we isolated UMNSAH/DF-1 cell line from *Gallus gallus*. For this, 9 ± 2 day old chicken embryo was taken. This was followed by the extraction of nicotine (1 mg/ml) from cigarette. The cells were then given nicotine stress and were observed for blackening after 24 h of incubation under 40× resolution of microscope. It was found that this blackening of the cells was permanent even after a wash with 1× phosphate-buffered saline (PBS) followed by replenishing the medium. The phytochemicals extracted were from the dried powder, which included *Curcuma longa* (薑黃 Jiāng Huáng; Turmeric) 40 mg/ml, *Azadirachta indica* (neem) 50 mg/ml, *Cinnamomum tamala* (bay leaf) 30 mg/ml, *Camellia sinensis* (綠茶 Lǜ Chá; Green Tea) 100 mg/ml, and *Ocimum sanctum* (tulsi) 30 mg/ml. When applied to nicotine-stressed cells, it was observed that ursolic acid in neem recovered 70%, followed by 65% recovery by tulsi (having triterpenoid), 50% recovery by the catechins in *Ca. sinensis*, and very little recovery shown by *Ci. tamala*. Due to the yellow coloration of the cells by *Cu. longa*, much could not be inferred, although it was inferable that it had resulted in little effects. Mixtures of these phytochemicals were used, and it was found that neem: tulsi diluted in 3:1 ratio was highly effective and cell recovery was almost 80%. 68% was recovered by tulsi: green tea in a ratio 1:3 and 42% by turmeric:green tea in a ratio of 1:5.

Key words: Nicotine, Oxidative phytochemicals, UMNSAH/DF-1

INTRODUCTION

Nicotine is an alkaloid found in the nightshade family of plants (Solanaceae) and has been found to be a potent parasympathomimetic and a stimulant drug. It is synthesized in the roots of the tobacco plant and stored in its leaves, and it constitutes around 0.6-3.0% of the dry weight of tobacco.^[1] An average cigarette contains 1 mg of absorbable nicotine,^[2] which acts as a stimulant in mammals. Nicotine has been found to inhibit chromatin-modifying

enzymes and class I and II histone deacetylases.^[3] It increases the levels of several neurotransmitters by binding to nicotinic acetylcholine receptors, with a higher affinity in the brain than in the skeletal muscles.^[4] Tobacco smoke contains normicotine, anabasine, and anatabine, along with monoamine oxidase inhibitors.^[5] Nicotine has a half-life of 2 h, and is therefore easier to analyze.^[6] When a cigarette is smoked, nicotine-rich blood passes from the lungs to the brain within 7 s and immediately stimulates the release of many chemical messengers such as acetylcholine,

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norepinephrine, epinephrine, vasopressin, histamine, arginine, serotonin, dopamine, autocrine agents, and β -endorphin.^[7] The side effects of nicotine include increased blood clotting tendency, bronchospasm, tremor and pain in muscles, dried mouth, nausea, dyspepsia, headache, light-headedness, dizziness, sleep disturbance, increased blood pressure, tachycardia, coronary artery constriction, hyperinsulinemia, and insulin resistance.^[8]

In this study, phytochemicals were extracted from five different plants, *Ocimum sanctum* (tulsi), *Azadirachta indica* (neem), *Cinnamomum tamala* (bay leaf), *Camellia sinensis* (綠茶 Lù Chá; Green Tea), and *Curcuma longa* (薑黃 Jiāng Huáng; Turmeric). Tulsi is considered to be an adaptogen, balancing different processes in the body, and is helpful for adapting to stress.^[9] It contains oleanic acid which is a naturally occurring triterpenoid acting as a powerful inhibitor of cellular inflammatory processes. Tulsi also has ursolic acid which decreases proliferation of cancer cells and induces apoptosis.^[10] Neem is found to have the same effects as tulsi. Tamala leaves contain caryophyllene oxide which undergoes reduction and produces β -caryophyllene. It is also found to have anti-inflammatory, antioxidant, antiulcer, and anticarcinogenic effects. When nicotine (1 mg/ml) extracted from tobacco was added as a stressor, all these compounds showed stress-relieving effect on cells and promoted growth and differentiation of UMNSAH/DF-1 AT cell.

MATERIALS AND METHODS

Nine-day-old fertilized egg was obtained from Midnapore (West Bengal, India). T25 flasks were procured from Bio-Rad (Gurgaon, India). Medium A and Medium B were obtained along with chicken fibroblast isolation kit (Medox, Bangalore, India). CO₂ incubator was from Thermo Fisher Scientific (Mumbai, India). All other equipments including micropipette were obtained from Amity Institute of Biotechnology (Noida, India).

Dissection and disaggregation of 9-day-old chicken embryo

A mark was made on the pointed side of the egg shell. The handle of a sterilized surgical scissors was used to give short gentle blows and then the egg was cut through the mark. Sterile forceps were used to separate the egg shell. Through the small

opening, the embryo was dropped on a sterile Petri plate, followed by separating the embryo from the other yellow mass. The embryo was washed with sterile distilled water twice and kept on a fresh sterile Petri plate for dissection. The head followed by the small tail was cut with a sharp sterile scalpel and then the body was split open as shown in Figure 1. The tissue was washed five times with phosphate-buffered saline (PBS) buffer which was diluted from a 10 × stock. The tissue was cut to small pieces, which was followed by its disaggregation using a sterilized mesh. Following the disaggregation of the tissue, the cells were passed through a 5 ml disposable syringe for further disaggregation. This step was repeated five times to yield loosened separate cells.

Isolation of cells and primary culture

The loosened mashed cells were taken and 10 ml of Medium A consisting of trypsin solution (0.25%) prewarmed at 37°C (supplied along with chicken fibroblast isolation kit; Medox) was added and kept in a T25 flask. The cells were incubated at 37°C in the presence of 5% CO₂ for 20 min. The supernatant was poured into a centrifuge tube with 10% fetal bovine serum and stirred for 10 min continuously. The cell suspension was taken and centrifuged at 1000 rpm for 10 min. The cell pellet was mixed with 20 ml of Medium B which consisted of Dulbecco's Minimal Essential Medium supplemented with 10% fetal bovine serum along with 0.0025 g Ampicillin (supplied with chicken fibroblast isolation kit; Medox). The cells were incubated at 37°C in the presence of 5% CO₂ for 20 min. The cell suspension was taken and centrifuged at 1000 rpm for 10 min. The cell pellet was mixed in 20 ml of Medium B along with 0.0025 g Ampicillin (supplied with chicken fibroblast isolation kit; Medox) and was distributed in four T25 flasks with each having 22,000 cells per cm², calculated using a hemocytometer. Five milliliters of Medium B along with 0.0025 g Ampicillin was hence supplemented in each flask. The flasks were incubated at 37°C in 5% CO₂ for 7 days.

Sub-culturing and seeding of cells

2.5% Trypsin in PBS buffer supplemented with 0.5 M ethylenediaminetetraacetic acid (EDTA) was used for disaggregation of the cells from the T25 flasks. For this purpose, warm trypsinization

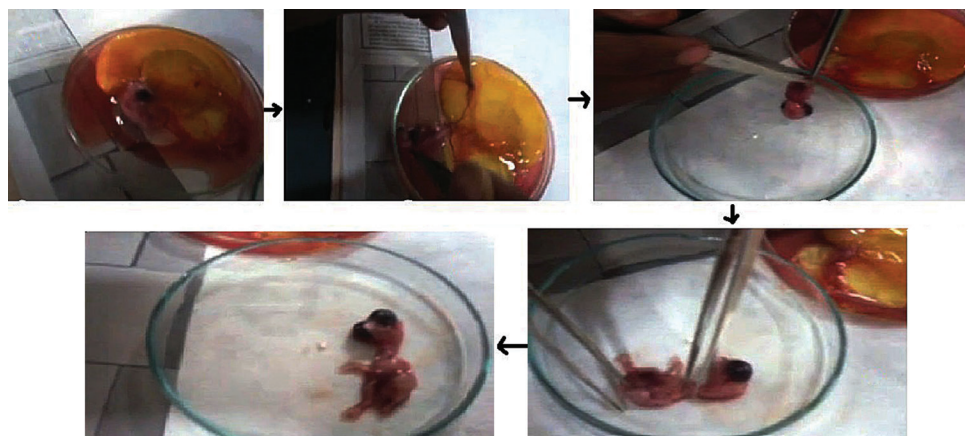


Figure 1. Stepwise extraction of the 9-day-old chicken embryo from the shell

method was followed. Trypsin, as prepared above, was added and final flask volume was made up to 25 ml. The T25 flasks were then kept for 30 min at 37°C in the presence of 5% CO₂. Intermittent stirring was also carried out for proper mixing of the trypsin. The cells that were suspended in medium and trypsin were centrifuged at 1000 rpm for 10 min. The supernatant was decanted and the cells were washed with PBS buffer twice and centrifuged in between. The cells were suspended in 20 ml of Medium B along with 0.0025 g Ampicillin. Exactly 22,000 cells per cm² were added to each T25 flask. Five milliliters of Medium B was seeded. Further sub-culturing was continued to keep the viable cells for the experiments.

Extraction of nicotine

Two grams of raw, cured tobacco was taken from an 84 mm cigarette, and boiled in 5 ml of sterile distilled water and filtered using a 0.45 µm Millipore filter. The extract was stored at room temperature in an aseptic place.

Culturing of cells in 4X3 culture plate

In each of the wells of A1–4, B1, and C1, 100 µl of the cells was added (3000 cells per cm² were seeded). This was followed by supplementation with 900 µl of Medium B along with 0.0025 g Ampicillin as shown in Figure 2. The culture plate was incubated for 3 days at 37°C in 5% CO₂, and almost 70% confluency was achieved.

Nicotine stress

The extracted nicotine was diluted with Medium B and was added to each of the wells where confluency had been achieved. A 1:1 dilution of nicotine and Medium B was done in which 500 µl of nicotine and 500 µl of Medium B were added. The culture plate was left for 24 h incubation at 37°C in 5% CO₂.

Preparation of extracts of phytochemicals

Preparation of the extracts was started by weighing the dried powder. The powder was mixed in 5 ml of sterile distilled water and boiled. The mixture was strained and filtered through a 0.45 µm Millipore filter. This was stored at room temperature in an aseptic place. For *Ci. tamala* (bay leaf), 150 mg of

the dried leaves was taken. For *Ca. sinensis* (綠茶 Lù Chá; Green Tea), 500 mg was taken. *Cu. longa* (薑黃 Jiāng Huáng; Turmeric) roots were taken, cut into small pieces, dried, and crushed, and 200 mg of the powder was taken. For *A. indica* and *O. sanctum*, 250 mg and 150 mg of the dried powder was used, respectively.

Addition of single phytochemicals to nicotine-stressed cells

One milliliter of the medium was discarded from each of the wells. Eight hundred microliters of *O. sanctum* and 1200 µl of Medium B along with 0.0025 g Ampicillin were added to well A4. One thousand microliters of *Ci. tamala* and 1000 µl of Medium B along with 0.0025 g Ampicillin were added to well A3. Six hundred microliters of *Ca. sinensis* and 1400 µl of Medium B along with 0.0025 g Ampicillin were added to well B1. One hundred microliters of *Cu. longa* and 1900 µl of Medium B along with 0.0025 g Ampicillin were added to well C1. A positive control was set in well A1 where 200 µl of fresh nicotine was mixed with 800 µl of Medium B along with 0.0025 g Ampicillin. Also, 1200 µl of *A. indica* and 800 µl of Medium B along with 0.0025 g Ampicillin were added to well A2, and 800 µl of *A. indica* and 1200 µl of Medium B along with 0.0025 g Ampicillin were added to well B2. After every 24 h of incubation, 500 µl of medium was discarded from each well, followed by replenishing with 500 µl of Medium B with 0.0025 g Ampicillin. Observations were made under 40× resolution of inverted light microscope.

Addition of combinations of phytochemicals to nicotine-stressed cells

To the well C3 were added *A. indica*: *O. sanctum* in a ratio of 3:1, i.e. 900 µl of *A. indica* and 300 µl of *O. sanctum*, with 800 µl of Medium B along with 0.0025 g Ampicillin. To the well B3 were added *A. indica*: *O. sanctum* in a ratio of 1:1, i.e. 500 µl of *A. indica* and 500 µl of *O. sanctum*, with 1000 µl of Medium B along with 0.0025 g Ampicillin. To the well C4 were added *Cu. longa*:*Ca. sinensis* in a ratio of 1:5, i.e. 100 µl of *Cu. longa* and 500 µl of *Ca. sinensis*, with 1400 µl of Medium B along with 0.0025 g Ampicillin. To the well B4 were added *O. sanctum*: *Ca. sinensis* in a ratio of 1:3, i.e. 300 µl of *O. sanctum* and 900 µl of *Ca. sinensis*, with 800 µl of Medium B along with 0.0025 g Ampicillin.

Determination of recovery ratio

The cells were washed with 1× PBS, and Gas Chromatography (GC) Nicotine Assay Method was carried out. To the cell wash, 1 ml of propanol was added. GC was carried out in Allele Life Sciences Pvt Ltd, Noida. *Column specification*: Heliflex AT-1 dimethylpolysiloxane capillary column – 30 m × 0.53 µm × 5 µm. *Temperature specification*: 150°C for 1 min, ramp 10°C/min to 260°C, 260°C for 18 min, 15 min equilibration. Analysis time was set for 30 min with 0.5 µl splitless injection and helium being the carrier gas. 1.05 mg/ml nicotine dissolved in methanol was used as the standard. The integrated peak area was used to calculate the nicotine content per microliter of the sample. The same assay was performed after subjecting the cells to phytochemicals and the percent recovery was calculated based on the following formula:



Figure 2. A 4×3 culture plate showing the addition of nicotine to wells A1–4, B1, and C1

$$\text{Percentage recovery} = \left(\frac{\text{Initial nicotine concentration} - \text{Final nicotine concentration}}{\text{Initial nicotine concentration}} \right) \times 100\%$$

RESULTS AND DISCUSSION

When nicotine was added to the cells, the positive control (A1) showed browning of the cells after 24 h of incubation when observed under 40× resolution of inverted light microscope. Also, the cell membrane started to blacken which could be distinctly seen in Figure 3. A few of the cells also died and, therefore, got detached and started to move when a little tap was given to the culture plate. Further, dark black blotches or spots started to appear at various places. Upon further incubation for 24 h, there was complete browning of the cells [Figure 4] with distinct black cell membranes. Also, almost one-third of the plate was completely covered with the black blotches or spots where the effect of nicotine was found to be stronger. Similar was the case in all the wells where nicotine was added. The negative control [Figure 5] was

made by culturing the pure cells, which were not subjected to nicotine stress or the addition of any phytochemical extract. No sign of blackening was observed. Also, there was no blackening of the cell membrane or browning of the cells. This gives clear evidence that nicotine deposition had taken place in the cell and the initiation of cell death is also responsible for it.

Among the cells to which *A. indica* (neem) extract was added [Figure 6], 70% could be recovered and prevented from the browning coloration. This was confirmed by the observation of the cells under a hemocytometer. In case of cells with *O. sanctum* (tulsi) extract [Figure 7], 65% could be recovered. Further, 50% was recoverable in case of *Ca. sinensis* (綠茶 Lù Chá; Green Tea) [Figure 8] and very little in case of *Ci. tamala* (bay leaf) [Figure 9]. *Cu. longa* (薑黃 Jiāng Huáng; Turmeric) gave the cells a natural yellow stain [Figure 10] and, therefore, was not quite distinguishable for its effect in curbing down the effect of nicotine.

In the cells where mixtures of phytochemicals were used, it was found that 3:1 diluted neem: tulsi [Figure 11] was highly effective over 1:3 diluted neem: tulsi and cell recovery was almost 80%. This was followed by tulsi: green tea in a ratio of 1:3 [Figure 12] and the least effect was shown by turmeric: green tea in a ratio of 1:5 [Figure 13].

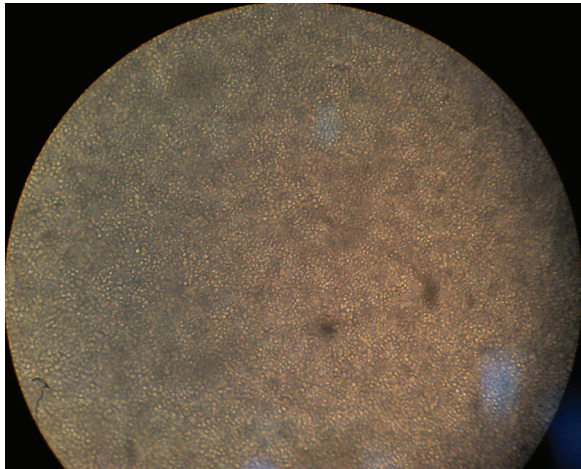


Figure 3. Nicotine-stressed cells after 24 h of exposure

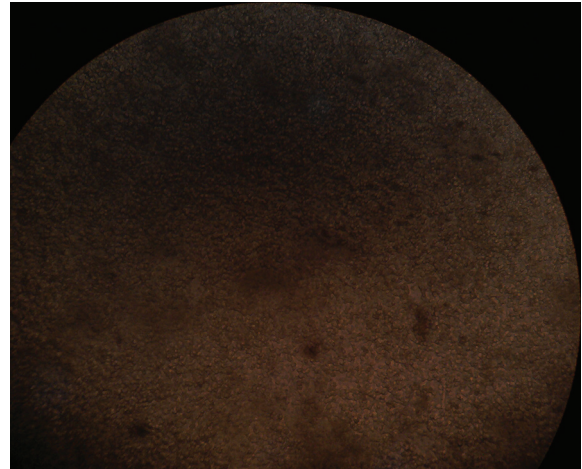


Figure 4. Positive control – nicotine-stressed cells after 48 h of exposure

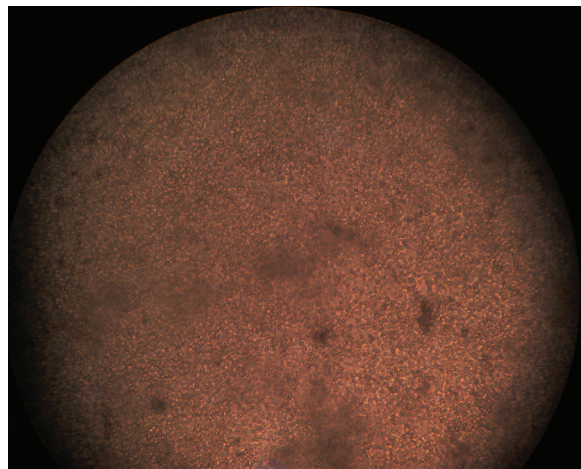


Figure 5. Negative control – cells without nicotine exposure

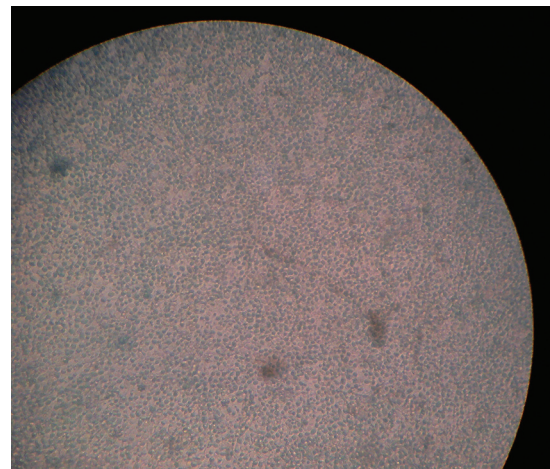


Figure 6. Nicotine-stressed cells with *A. indica*

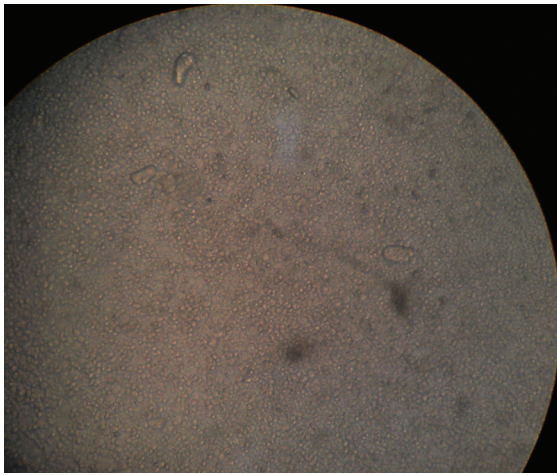


Figure 7. Nicotine-stressed cells with *O. sanctum*

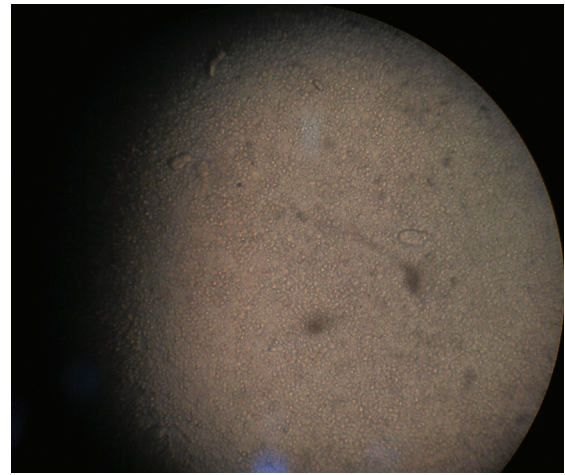


Figure 8. Nicotine-stressed cells with *Ca. sinensis*

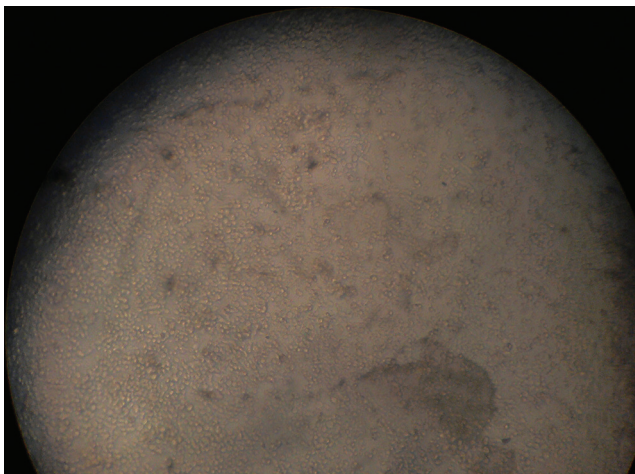


Figure 9. Nicotine-stressed cells with *Ci. tamala*

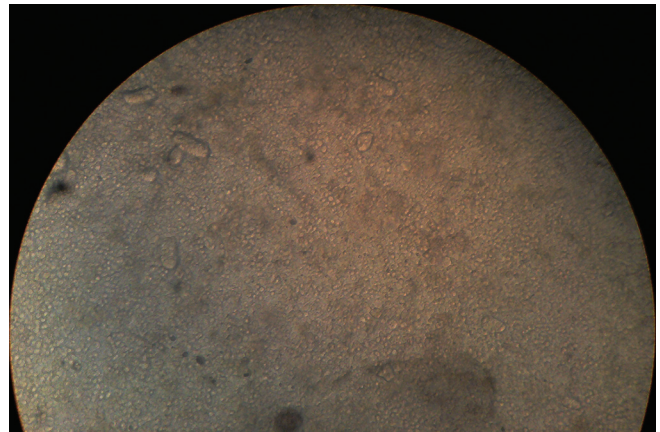


Figure 10. Nicotine-stressed cells with *Cu. longa*

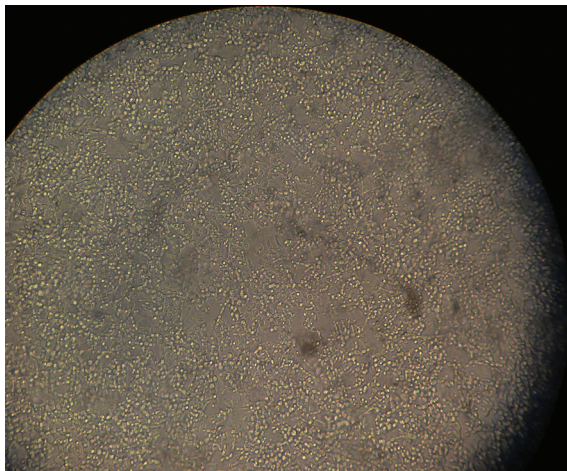


Figure 11. Nicotine-stressed cells with *A. indica* and *O. sanctum* added in the ratio of 3:1

Fibroblast cell membrane is rich in polyunsaturated fatty acids. The attachment of peroxidants to membrane can result in hemolysis of cell.^[10] Cigarette smoke increased 2,2'-azo-bis-(2-amidino-propane) dihydrochloride-induced membrane hemolysis.^[11]



Figure 12. Nicotine-stressed cells with *O. sanctum* and *Ca. sinensis* added in the ratio of 1:3

Ursolic acid in *A. indica* was found to be a weak aromatase inhibitor. Aromatase, also called estrogen synthetase or estrogen synthase, catalyzes many reactions involved in steroidogenesis, due to which it results in a complete reversal of plasma membrane transbilayer sterol distribution. Triterpenoids are terpenoid derivatives of triterpenes such as sterols, cholesterol, etc., found in *O. sanctum* (tulasi). The addition of neem: tulasi extract in 3:1

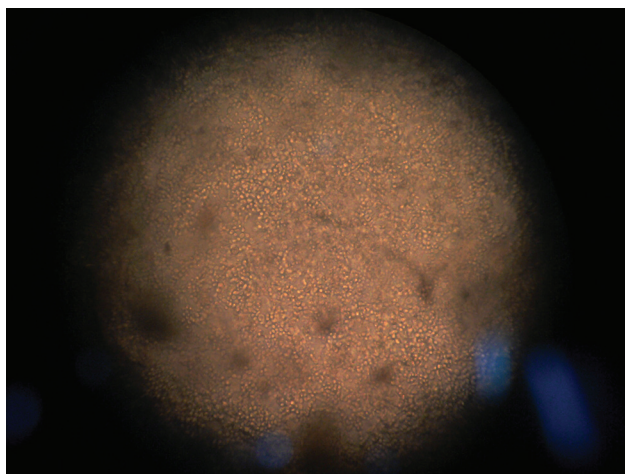


Figure 13. Nicotine-stressed cells with *Cu. longa* and *O. sanctum* added in the ratio of 1:5

ratio suited best for inhibiting the steroidogenesis reaction, hence stopping the attachment of peroxidant molecules to the membrane and minimizing hemolysis. As ursolic acid is a weak inhibitor, it is needed in an appropriate ratio. Therefore, neem is added to tulsi in a ratio of 3:1. Catechin is a flavan-3-ol, a type of natural phenol and a weak antiperoxidant present in green tea (*Ca. sinensis*). Hence, it is also added in 3:1 ratio with tulsi extract. Turmeric contains curcuminoids (diferuloylmethane), demethoxycurcumin, and bisdemethoxycurcumin which are antioxidants; but for proper visualization of cells, we added turmeric in a ratio of 1:5 with green tea. The basic idea though was to get the best result for recovery of the nicotine-stressed cells.

The remarkable recovery of cells from nicotine stress using the combinations of phytochemicals is the first of its kind and, hence, is significant in the preparation of therapeutic drugs for usage in *in vivo* systems.

CONCLUSION

When the five extracted phytochemicals were applied to nicotine-stressed cells, it was observed that ursolic acid in neem recovered 70%, followed by 65% recovery by tulsi (having triterpenoid), 50% recovery by catechins in *Ca. sinensis* (綠茶 *Lù Chá*), and very little recovery by *Ci. tamala*. Due to the yellow coloration of the cells by *Cu. Longa*, much could not be inferred, although it was inferable that it had resulted in little effects. Mixtures of these phytochemicals were used and it was found that neem: tulsi diluted

in 3:1 ratio was highly effective and cell recovery was almost 80%. This was followed by tulsi: green tea in the ratio of 1:3 and the least effect was shown by turmeric: green tea in a ratio of 1:5.

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