2nd Annual Convention of

North East (India) Academy of Science and Technology (NEAST)

&

International Seminar on Recent Advances in Science and Technology (ISRAST)

(16th -18th November 2020)

(Virtual)

11215150220

Certificate

This is to certify that

Mr./Ms./Dr./Prof. Puja Pandey

Department of Biotechnology, Mizoram University, Tanhril, Aizawl–796004 (India)

has attended and presented an Oral Presentation entitled, "Bacterial Antioxidant Enzyme Activity in Response to Reactive Oxygen Species induced by Tuibur Stress" in the 2nd Annual Convention of North East (India) Academy of Science and Technology (NEAST) & International Seminar on Recent Advances in Science and Technology (IRSRAST) during 16th-18th November 2020 (Virtual) organized by NEAST, Mizoram University, Aizawl-796004, Mizoram (India) .

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General Secretary, NEAST

Prof. R. Lalthantluanga
President. NEAST

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28.01.2021

TO WHOM IT MAY CONCERN

This is to certify that Ms. Puja Pandey, a M.Phil. scholar Registration No. MZU/M.Phil./625 of 12/06/2020 has worked on the thesis entitled, 'Tuibur stress-induced antioxidant enzyme and mutation profiling in bacteria' has fulfilled all criteria prescribed by the UGC (Minimum Standard and Procedure governing M.Phil. Regulations). She has fulfilled the mandatory publications (enclosed). It is also certified that the scholar has been admitted in the Department through an entrance followed by an interview as per UGC Regulation 2016.

Yours faithfully

(N. Senthil Kumar)

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TUIBUR STRESS-INDUCED ANTIOXIDANT ENZYME AND MUTATION PROFILING IN BACTERIA

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY

PUJA PANDEY

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DEPARTMENT OF BIOTECHNOLOGY

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JANUARY 2021

TUIBUR STRESS-INDUCED ANTIOXIDANT ENZYME AND MUTATION PROFILING IN BACTERIA

BY

PUJA PANDEY

Department of Biotechnology

Name of the Supervisor: Prof. N Senthil Kumar

Submitted

In partial fulfillment of the requirement of the Degree of Master of Philosophy in Biotechnology of Mizoram University, Aizawl

CERTIFICATE

This is to certify that the dissertation entitled "**Tuibur stress-induced antioxidant enzyme and mutation profiling in Bacteria**" submitted to the Mizoram University; in partial fulfillment for the degree of M.Phil. of Philosophy in Biotechnology is a record of research work carried out by **Miss Puja Pandey** under our personal supervision and guidance.

No part of this dissertation has been reproduced elsewhere for any degree.

Dated: 29/01/2021

Dr. N. Senthil Kumar

DECLARATION
Mizoram University
January 2021

I, **Puja Pandey**, hereby declare that the subject matter of this dissertation entitled "**Tuibur stress-induced antioxidant enzyme and mutation profiling in Bacteria**" is the record of work done by me, that the contents of this dissertation did not form basis of the award of any previous degree to me or to the best of my knowledge to anybody else, and that the dissertation has not been submitted by me for any research degree in any other University/Institute.

This is being submitted to the Mizoram University for the degree of Master of Philosophy in Biotechnology.

Date: 29/01/2021

(Candidate)

(Dr. N. Senthil Kumar)

(Dr. N. Senthil Kumar)
(Supervisor)

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Date: 29/01/2021 PUJA PANDEY

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INTRODUCTION

Tobacco is produced from a genus of herbs *Nicotiana* and is used in many forms, including smoke and smokeless. In India, a wide range of smokeless tobacco products are used (Bhonsle *et al.,* 1992). The alkaloid nicotine, a secondary metabolite, is the main active component of tobacco and is responsible for its drugs and calming properties.

Previous studies reported a very high prevalence of tobacco use among men and women in Mizoram and Manipur, and this could be attributed to the widespread cultural acceptance of the use of tuibur in the population (Sinha *et al.,* 2004). An undescribed tobacco product called “tuibur” was found to be used in Mizoram during the Global Youth Tobacco Survey (Sinha *et al.,* 2004). The smokeless tobacco product, which is a unique aqueous concentrate of tobacco smoke is known locally as tuibur. A cultural tradition has been the use of tobacco in this particular form. The habitual intake of tuibur is believed to be one of the "safe" means of delivery of nicotine. This product is generated by tobacco smoldering. Stalk (stem, midrib) is percolated as alkaline solution through the tobacco ash (feedstock) until the preparation turns the color to cognac and has a pungent smell of nicotine (Madathil *et al.,* 2018). In vitro studies with the use of the allium, root tests showed the toxic nature of tuibur (Mahanta *et al.,* 1998). It is commercially made in various towns and villages across Mizoram using tobacco stalk (stem, midrib) normally procured from Myanmar, across the international border. Indigenous crude devices are used to render tuibur on an industrial-scale cottage. As a sip, consumers take roughly 5 to 10 mL 'alkaline' tuibur solution and keep it for a few minutes in the lower oral space and then spit it out. The majority of users use it many times in a day. The use of Tuibur is prevalent in both smokers and non-smokers and may be one of the significant reasons for the high prevalence of stomach cancer among the population in Mizoram (Phukan *et al.,* 2005; Mukherjee *et al.,* 2020; Chakraborty *et al.,* 2021).

It is understood that the use of smokeless tobacco or cigarette smoking induces different types of cancer because different chemical components of tobacco

are found to be carcinogenic. It is possible to describe a carcinogen as a chemical, physical or biological agent that causes cancer or induces cancer. Tobacco is known to contain different types of chemical species (hydrophilic and hydrophobic), many of which have been reported to have carcinogenic and cytotoxic properties (Yadav *et al.,* 2018). The damaging effect of tobacco may be attributed to its vast array of chemical compositions arising from the mode of preparation of a tobacco product. Heavy metals like arsenic, cadmium, chromium, lead, nickel, etc. present in tobacco have also been found to cause glomerular dysfunction (Lalruatfela *et al.,* 2017).

Cigarette tobacco use is linked with many disorders and tobacco products have been estimated to contain over eight thousand chemicals, of which about 69 are carcinogenic (Arimilli *et al.,* 2012). Some of the common carcinogenic chemicals found in tobacco include quinoline, nitrite, cadmium, lead, arsenic, nickel, chromium, hydrazine, benzo(a)pyrene, N' - nitrosonornicotine, N' - nitrosoanatabine, N'-nitrosoanabasine, 4-(methylnitrosamino)-1-(3-pyridyl)-1- butanone, N-nitrosodimethylamine, etc. (Talhout *et al.,* 2011). Tuibur also contains other trace heavy metals from tuibur using ICP-MS such as Cd, Zinc, Pb, Ni, As, Cu, Cr and Mn in tuibur (Lalmuanpuii, 2016; Muthukumaran, 2016).

In previous studies, it was also suggested that cadmium could also accentuate the production of ROS (Manca *et al.,* 1994; Bagchi *et al.,* 1997; Liu and Jan, 2000) which in turn causes elevation of lipid peroxidation, ultimately results in DNA damage (Liu *et al.,* 2009). An oxidative imbalance imposed by ROS in tissues and cellular components causing damage to membranes, DNA, and proteins is involved in the process of lead-induced oxidative stress (Patra *et al.,* 2011)

For several enzymes as well as other proteins, zinc serves as an important catalytic and structural cofactor, while zinc is also regulated by cell differentiation, proliferation, and gene expression. For several years, zinc deficiency has been known to cause increased oxidative stress and, as a result, increased oxidative damage to DNA, proteins, and lipids (Eide, 2011). Zinc gradually becomes toxic because of the excessive intake that is likely to be involved in causing pathological conditions that have been associated with oxidative stress (Ajab *et al.,* 2008). Nickel induces higher

levels of endogenous cellular hydrogen peroxide and its associated short-lived and hyper-reactive oxygen species (Lynn *et al.,* 1997; Das *et al.,* 2001). Nuclear protein damage caused by nickel reduces the enzyme activity needed for DNA replication, transcription, recombination, and repair (Das *et al.,* 2008). Through oxidative stress, arsenic is said to exhibit its toxicity by generating reactive oxygen species (Sharma *et al.,* 2006). Due to excessive intracellular accumulation, copper becomes poisonous and plays a role in initiating the apoptotic processes and generation of reactive oxygen species (Santon *et al.,* 2004). Reactive oxygen species have also been shown to be directly involved in oxidative damage to lipids, proteins, and DNA in arsenic-exposed cells, which can ultimately lead to cell death (Boulikas, 1991). Ingestion of chromium (VI) compounds exacerbates oxidative stress as DNA damage is induced by the generation of mainly hydroxyl radicals (OH•) (Liu *et al.,* 1999) and has possible effects on cell alteration (Petrilli *et al*., 1982).

Nicotine ([1-methyl-2-(3-pyridyl)] pyrrolidine), a major alkaloid synthesized as an L-isomer, is a significant toxic natural product in the tobacco plant. It acts as a mild stimulant that is perhaps linked with the generation of oxidative radicals, which in turn affects many cellular processes ranging from induction of gene expression to hormone secretion, in addition to regulation of enzymatic activities (Qiao *et al.,* 2005). Putative toxic effects of tuibur were also studied using a modified version of the Allium test. However, nicotine's putative effects on organisms have not been thoroughly elucidated in detail. Low levels of radicals can be beneficial, while toxicity is associated with high concentrations (Kovacic *et al.,* 2005).

Many pollutants are redox-active which leads to the generation of superoxide radical ($O_2^{-\bullet}$), the hydroxyl ($OH^{\bullet}$) radical, and hydrogen peroxide (Kang *et al.,* 2007). On the other hand, oxidative stress could be encountered by pollutant-degrading bacteria, both as a direct effect of the contaminants themselves and also from the transient species generated during the biodegradation processes (Park *et al.,* 2004).

The chemical compounds of tobacco that can lead to a distinct response in Gram-positive and Gram-negative bacteria and the complex mechanism is are still

not very clear (Buurman *et al.,* 2006). Between Gram-negative and Gram-positive strains, the levels of antioxidant enzymes differ markedly. Gram-positive strains do not respond to exposure to nicotine, while Gram-negative bacteria *E. coli* K12 and *Pseudomonas* sp. HF-1 showed higher nicotine stress sensitivity. Thus, Gram-negative bacteria are the more suitable organisms for the studies on environmental nicotine pollution (Yadwad *et al.,* 1990). There were various effects of nicotine on the different antioxidant enzymes of different bacteria. The oxidative stress responses to many impurities in bacteria have so far been extensively studied (Geckil *et al.,* 2003). The effects of nicotine on oxidative stress have also been substantially studied in mammalian cells (Lu *et al.,* 2004). Nicotine or tobacco and Tuibur can produce ROS which can induce higher oxidative stress in the culture and reduce glutathione level in cells and can inhibit catalase and glutathione activities (Aspera-werz *et al.,* 2018).

Chemicals containing Tuibur induce the generation of reactive oxygen species (ROS) in different cells, whereas the expression of cellular antioxidant enzymes such as Superoxide Dismutase (SODs), Catalase (CAT), Glutathione Reductase (GR), Peroxidase (POD), and glutathione-S-transferase (GST) can neutralize the toxic effects of ROS (Lalruatfela *et al.,* 2017). For bacterial cells, reactive oxygen species (ROS) can be harmful. The superoxide anion ($O_2^{\bullet}$) can be dismutated by SOD to hydrogen peroxide and oxygen and the hydrogen peroxide scavenging enzyme CAT eliminates hydrogen peroxide concomitantly. Meanwhile, in many intracellular physiological functions, ATPase plays an important role and can be considered as a sensitive toxicity indicator (biomarker) (Hidalgo *et al.,* 2004).

In the previous research, oxidative stress occurs when *Pseudomonas* sp. metabolizes naphthalene, nicotine, and other substrates and the stress can be minimized by antioxidant enzymes (ATPase, CAT, and SOD) (Kang *et al.,* 2007). Previous studies have documented that the SoxR regulon orchestrates genes in *E. coli* to protect against certain forms of oxidative stress through the SoxR-regulated SoxS transcription activator synthesis (Park *et al.,* 2006). SOD plays a significant role in resistance to nicotine stress in *E. coli* in comparison to other antioxidant enzymes. However, between *Pseudomonas* and *E. coli*, the genetic responses to

superoxide stress differs (Park *et al.*, 2006), and CAT is essential to *Pseudomonas* for the acquired antioxidant resistance (Elkins *et al.,* 1999). *Pseudomonas* species have more complex regulatory systems for nicotine-induced stress than the *E. coli* system (Venturi 2003).

In prokaryotic cells, during the metabolism of nicotine, reactive metabolites were released, that are not appropriately inactivated, may eventually form a covalent (irreversible) bond to cellular DNA leading to the formation of deleterious DNA adducts (Shao *et al.,* 2009). The 16S rRNA gene sequencing is used as a tool to identify bacteria at the species level and assist with differentiating between closely related bacterial species. Many laboratories rely on this method to identify unknown pathogenic strains (Woese, 1987, Achtman, 2002). The 16S rRNA gene encodes the small subunit ribosomal RNA molecules of ribosomes, responsible for the essential process of converting genetic messages to functional cell components via the translation of mRNA to proteins. The 16S rRNA gene consists of highly conserved nucleotide sequences, interspersed with variable regions that are genus- or species-specific.

The most common primer pair was designed by Weisburg *et al.* (1991) and is currently referred to as 27F and 1492R; however, for some applications, shorter amplicons may be necessary. In addition to highly conserved primer binding sites, 16S rRNA gene sequences contain hypervariable regions that can provide species-specific signature sequences useful for the identification of bacteria. The bacterial 16S gene contains nine hypervariable regions (V1–V9), ranging from about 30 to 100 base pairs long, that are involved in the secondary structure of the small ribosomal subunit (Gray *et al.,* 1984). Estimation of the ratio of the rates of transitions to transversions (Ti: Tv ratio) for a collection of aligned nucleotide sequences is important because it provides insight into the process of molecular evolution and such estimates may be used to further model the evolutionary process for the sequences under consideration (Strandberg *et al.*, 2003).

The purpose of this study is to provide data on the antioxidant enzymes such as CAT, GR, SOD and genetic alterations in DH5α *E. coli*, a gram-negative bacterium, treated with tuibur.

OBJECTIVE

1. To study the Antioxidant Enzyme Activity and gene mutations induced by Tuibur stress in Bacteria

MATERIALS AND METHODS

In this study, *Escherichia coli* strain DH5α (Gram-negative bacteria) was cultured at 37ºC at 120 rpm in liquid LB medium (Ausubel *et al.,* 1999). 5 g of NaCl, 5 g of peptone, and 2.5 g of yeast extract were added separately with 500 ml of milli Q water to the container. The mixture was boiled to dissolve and sterilized for 20 minutes at 15 psi, 121°C by autoclaving (Bertani, 2004). Tuibur was collected from the local manufacturing site in the Aizawl district, Mizoram. A high grade of Tuibur indicative of the higher quantity of nicotine (Lalmuanpuii *et al.,* 2016) present was used to treat the bacterial cells.

Tuibur treatment on bacterial strains

One ml bacterial strain was inoculated (~ 0.1 at OD_{600}) from overnight mother culture into 20 ml of sterile LB broth and incubated overnight for 24 h at 37ºC in 120 rpm. Bacterial cultures were sub-cultured to new LB broth after 24 h and kept for 1 h incubation at 37ºC in 120 rpm and after 1 h of incubation tuibur treatment (different concentration; i.e., 10%, 40%, 100% and 0.01% nicotine) were performed daily for 30 days during sub-culturing. The optical density was measured after 24 h at 600 nm. The bacterial cells were pelleted by centrifuging at 4000 rpm for 10 min at 4 ºC and the biomass of growth, total protein, and antioxidant enzymes were estimated every once a week for a month during the treatment schedule.

Cell lysate preparation

The cells were resuspended with chilled lysis buffer containing 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM DTT, 1 mM PMSF. The cell suspension was kept on ice for 10 min. 10 µl PMSF (100 mM) per ml of cell lysis buffer was added. Sonication was performed with 10 short bursts of 15 sec followed by intervals of 30 sec for cooling in ice. All the steps were carryout on ice. Avoid foaming and remove cell debris that was performed by centrifugation at 4°C for 10 min at 8000 rpm. Finally, the cell lysate was used for total protein ATPase and anti-oxidant enzyme estimation.

Assessment of Biochemical Parameters

To scavenge reactive oxygen species, cells were equipped with a very efficient antioxidative defence system that protects them from the destructive oxidative burst. Oxidative burst under stress was assessed by studying protein content and antioxidative enzyme activities viz. CAT, GR, and SOD in *E. coli* using UV-Visible Spectrophotometer.

Total protein estimation

Bradford reagent

50 mg of Coomassie Brilliant Blue G-250 was dissolved in 50 ml of methanol and 100 ml 85% (w/v) phosphoric acid (H_3PO_4) was added. The acid solution mixture was added slowly into 850 ml of distilled water until the dye dissolved completely. Whatman #1 paper filter was used to remove the precipitates just before use (Bradford, 1976).

Micro assay procedure (<50 µg ml-1 protein):

Five dilutions of protein standard BSA: 2, 4, 6, 8, 10 µg was prepared. 150 µl of each standard sample in duplicates was pipetted and 150 µl Unknown sample (bacterial cell lysate) to the well. 150 µl of dye reagent concentrate was added to each tube and mixed thoroughly (2 blanks set for the standard sample without adding the dye) and incubated at room temperature (RT) for at least 5-10min. Absorbance will increase over time; samples were incubated at room temperature for 1 h and absorbance was measured at 595 nm (Bradford, 1976).

Antioxidant Enzyme Estimation

Catalase (CAT) assay

The activity of catalase was determined according to the method of Aebi (1983). Catalase activity can be measured by following either the decomposition of hydrogen peroxide or the liberation of oxygen. The UV spectrophotometric

technique is the method of choice for biological material. In the ultraviolet range, Hydrogen peroxide shows a continual increase in absorption with decreasing wavelength. A decrease in extinction per unit time at 240 nm can be directly followed by the decomposition of Hydrogen peroxide. A catalase activity measure is a difference in extinction per unit time (Aebi, 1983).

Glutathione Reductase (GR) assay

Glutathione reductase catalyzes the reduction of glutathione disulfide (GSSG) involving the oxidation of NADPH. Catalytic activity is measured by following the decrease in absorbance due to the oxidation of NADPH. GR activity was calculated by measuring the oxidation of NADPH at 340 nm. The reaction mixture contains 1.8 ml phosphate buffer, 300 µl each of EDTA, NADPH, oxidized glutathione (GSSG), and enzyme extract. In a spectrophotometer, the readings were registered every 20 seconds at 340 nm against distilled water blank for a minimum of 4 minutes. The assay mixture without the extract served as the control to monitor the nonspecific binding of the substrates (Habig *et al.*, 1974).

Superoxide Dismutase (SOD) assay

The assay of SOD is based on the inhibition of the rate of reduction of cytochrome C by the superoxide radicals. The final concentrations of a 3 ml reaction mix are 50 mM potassium phosphate, 0.1 mM EDTA, 0.05 mM xanthine, 0.01 mM cytochrome C, 1 unit superoxide dismutase and 0.005-unit xanthine oxide. One unit will inhibit the rate of reduction of cytochrome c by 50% in a coupled system, using xanthine and xanthine oxidase at pH 7.8 at 25^{o}C in a 3 ml reaction volume. In a spectrophotometer, the readings were registered every 20 seconds at 340 nm for a minimum of 4 minutes (Singh, 2007).

Statistical Analysis

SPSS 22, GraphPad-9, R kit, and Microsoft Excel 2013 were used to conduct the statistical analysis. The student's t-test was used to determine the significant difference among the treatment groups (Mishra *et al.*, 2019). Duncan's test was

employed to determine the significant difference between overall factors according to no of days (Bewick *et al.*, 2004).

Bacterial DNA isolation

Overnight cultures of the tuibur treated bacterial strain were grown in liquid LB medium. The DNA was extracted by maxiprep phenol extraction and ethanol precipitation method (Ausubel *et al.,* 1999). The quality and quantity of extracted DNA were analyzed by agarose gel (0.8%) electrophoresis and the DNA yield was quantified using a Qubit® 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA).

PCR amplification and Sequencing

The DNA regions containing the 16S rRNA gene was PCR amplified using oligonucleotide primers. The isolates were subjected to amplification of 16S rRNA gene by using universal primers [forward 16S primer (27F)- 5' AGAGTTTGATCMTGGCTCAG 3' and reverse primer (1492R) – 5' GGTTACCTTGTTACGACTT 3'] (Heuer *et al.,* 1997). Reactions were performed in a total volume 10 µl consisting 1 µl genomic DNA, 0.3 µl of each primer, 1.2 µl deoxynucleotide triphosphate, 1 µl of PCR buffer, o.2 µl of taq DNA polymerase and 6.5 µl of milli Q water. PCR was performed under the following conditions: initial denaturation step at 95^0C for 5 min, followed by 30 cycles of denaturation at 95^0C for 1 min, annealing at 52.5^0C for 1 min and extension at 72^0C for 1.3 min with the final extension step at 72^0C for 7 min (Eppendorf, Germany). The amplified products were analyzed by electrophoresis through 1.5% agarose gel made in 1X TAE buffer and. The PCR bands were analyzed documented using Gel documentation system (BioRAD, USA) and were purified by the Pure Link PCR purification kit, and sequenced by the Sanger methodology with the Big Dye Terminator v 3.1 Automated Cycle Sequencing using ABI 3500 instrument according to manufacturer protocol (Thermo Scientific USA). All the sequences containing the mutation were evaluated for their potential pathogenicity using the following algorithms: DNA baser version 4.0 (dos Santos *et al.,* 2019).

Sequencing analysis

All the sequencing data were edited with Geospiza's FinchTV 1.4 (Treves, 2010) and are analyzed by MEGA X, Clustal Omega (Kimura, 1980; Tamura *et al.,* 2004; Kumar *et al.,* 2018). Sequence comparison packages such as BLAST (Ndayambaje *et al.,* 2019), Clustal Omega are used for the alignment of several 16s rRNA gene sequencing. The relation between bacterial species studied by the construction of phylogenetic tree using MEGA X.

16S rRNA secondary structure was predicted from DNA sequences of 16S rRNA using an RNAfold web server. In this study, Using the RNAfold web server **(**in silico tool), the stem-loop structure of 16S rRNA along with its three-dimensional conformation was analyzed (Singh *et al.,* 2018).

RESULT

Estimation of *E. Coli* Biomass

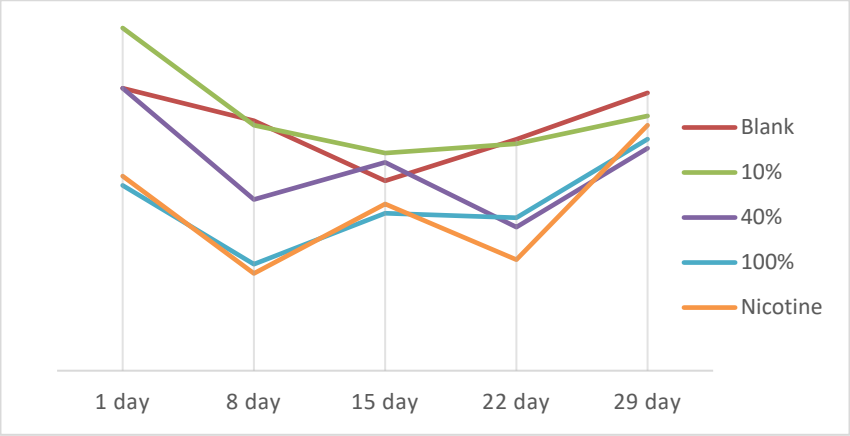

Fig. 1. Estimation of total Biomass in *E. coli* (day wise).

(The cells treated with tuibur (10%, 40%, and 100%) and blank, Nicotine (0.01%) was used as a positive control)

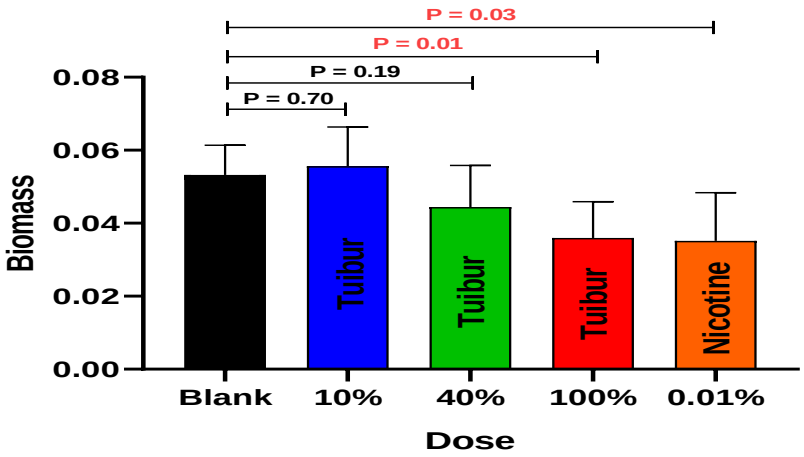

Fig. 2. Estimation of total Biomass in *E. coli.*

The *E. coli* cells were treated with different concentrations (10%, 40%, and 100%) of tuibur and Nicotine (0.01%) as positive control for 24 h daily for 29 days in a continuous manner. Untreated cells (Blank) were used as a control for the identification of tuibur toxicity in bacteria. We observed relatively higher biomass in Blank and 10% tuibur, relatively moderate biomass in 40% tuibur, and Low biomass

on 100% tuibur and positive control samples throughout the study period. But, the biomass of all the samples increased at end of the study (29 days) (Fig. 1). In overall comparison between day, control (blank) and tuibur (10%, 40%) treated decreased on the 29th day compared to the 1st day. On the other hand, 100% tuibur and nicotine (+ control, 0.01%) treated increased in 29th day compared to the 1st day (Fig.1). The biomass was directly proportional to the concentration of tuibur. Overall estimation of biomass yield culture treated with 100% tuibur ($p=0.01$) and nicotine ($p=0.03$) showed significance when compared with the control (Fig.2).

Estimation of total protein in *E. coli*

We observed a relatively higher amount of total protein in Blank and 10% tuibur, a relatively low amount of total protein in 40% tuibur, and a moderate amount of total protein in 100% tuibur as well as positive control samples throughout the study period. But, at end of the study (29 days), the amount of total protein of all the samples were relatively equal (Fig. 3). There is a continuous increase in the protein content in 40% tuibur treated culture from the day 15 compared to the day1. Though nicotine treatment was showing the same trend after the day 22, the total protein amount (mg/ml) was drastically decreasing. According to day wise analysis, no significance was found (fig.3). Overall estimation of total protein compared with the control; culture treated with 40% tuibur ($p=0.01$) showed significance (Fig.4).

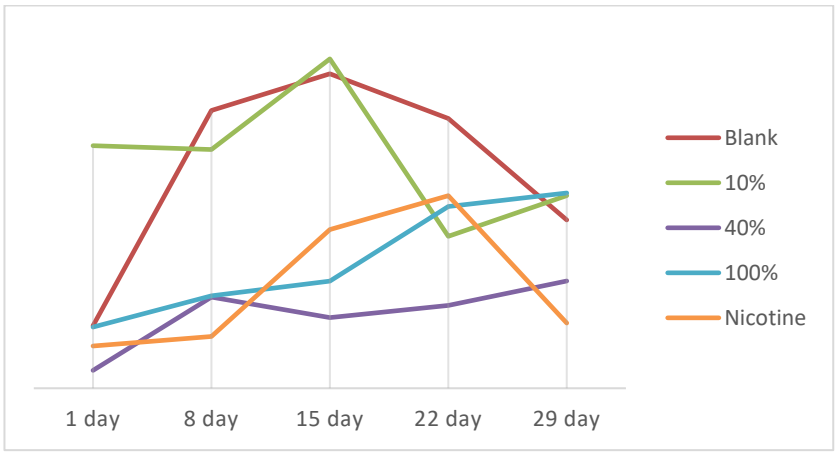

Fig. 3. Estimation of total protein in *E. coli* (day wise).

(The cells treated with tuibur (10%, 40%, and 100%) and blank, Nicotine (0.01%) was used as a positive control)

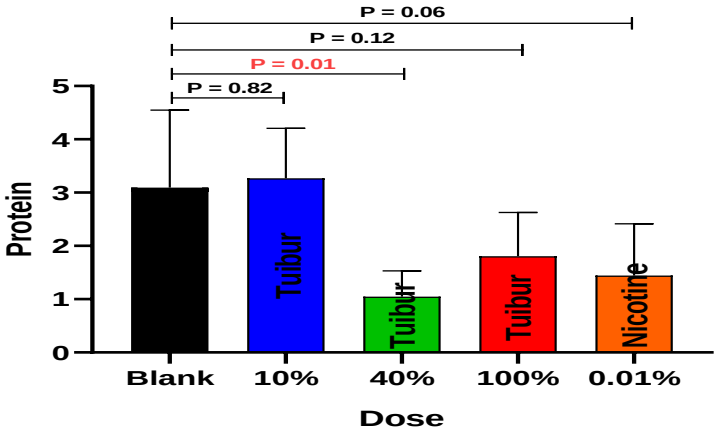

Fig. 4. Estimation of total protein in *E. coli*.

Estimation of Catalase activity in *E. coli*:

We observed a dose-dependent manner of catalase activity in the initial period of treatment (1-7 days). On day 8, the culture treated with 100% tuibur showed a significant increase in the catalase activity than compared with the blank and nicotine. After the 8th day, the catalase activity of 100% treated culture significantly decreasing. The activity of catalase in day 1 and 8 showed significance

compared with day 15 and 22. On day 29, the catalase activity showed the most significant value compared with the other days (fig.5).

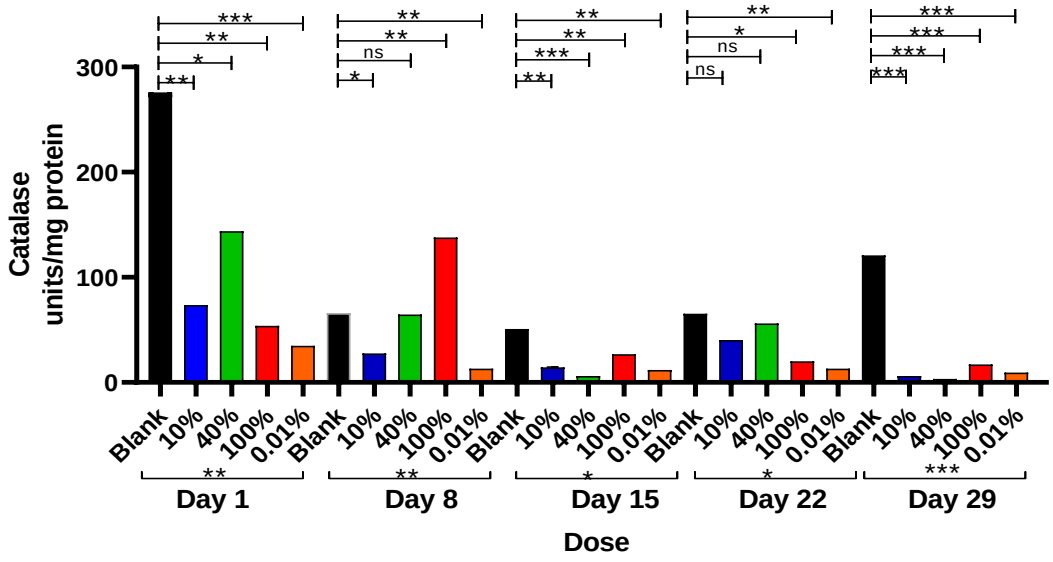

Fig. 5. Estimation of total Catalase activity in *E. coli*.

(The cells treated with tuibur (10%, 40% and 100%) and blank, Nicotine (0.01%) was used as a positive control) (*= 0.05, **= 0.001, ***= 0.0001, ns = not significant)

Estimation of total Glutathione reductase activity in *E. coli*:

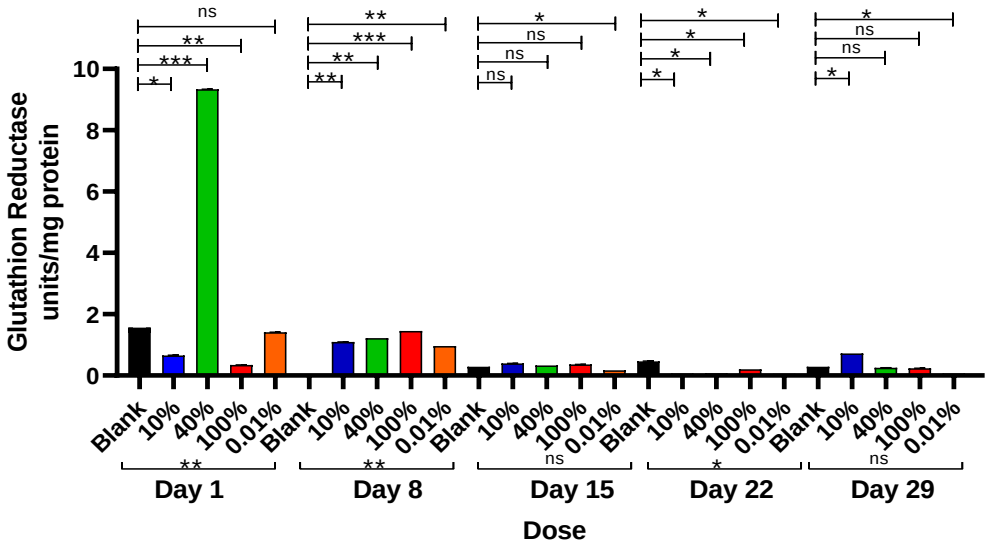

Fig. 6. Estimation of total Glutathione reductase activity in *E. coli*.

(The cells treated with tuibur (10%, 40% and 100%) and blank, Nicotine (0.01%) was used as a positive control) (*= 0.05, **= 0.001, ***= 0.0001, ns = not significant)

The activity of glutathione reductase (GR) gradually decreased on day 8 and after that, it decreased drastically. Compared to the day 15 and 29, the day 1, 8 and 22 showed significance (fig.6).

Estimation of total Superoxide Dismutase activity in *E. coli*.

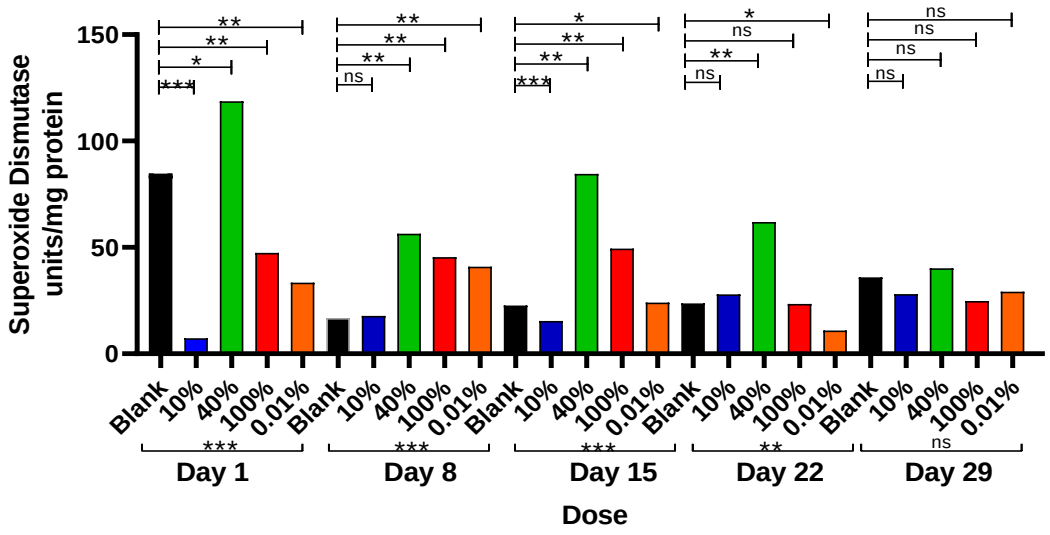

Fig. 7. Estimation of total Superoxide Dismutase activity in *E. coli*.

(The cells treated with tuibur (10%, 40% and 100%) and blank, Nicotine (0.01%) was used as a positive control) (*= 0.05, **= 0.001, ***= 0.0001, ns = not significant)

SOD activity decreased steadily during the study period. Compared to the day 29, the days 1, 8 and 22 were significant. High significance was seen on day 1st, 8^{th,} and 15th (fig.7).

PCR amplification

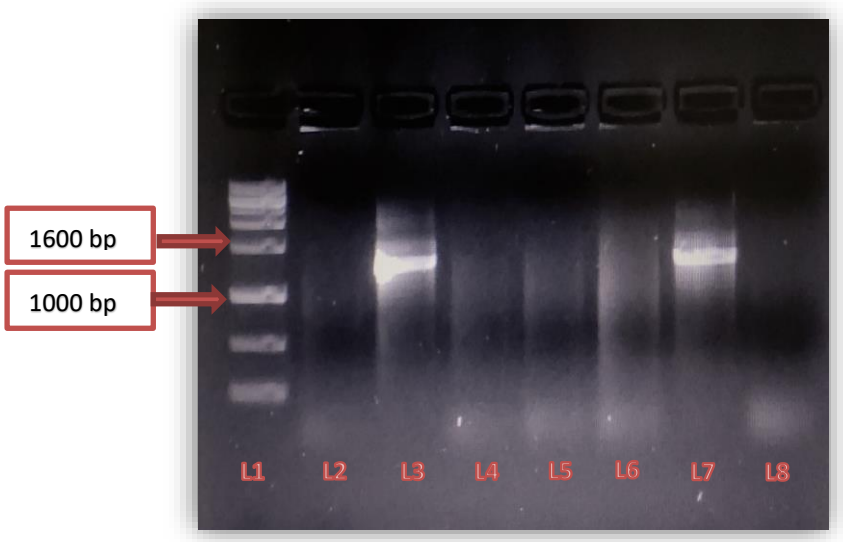

Fig.8. Representative gel electrophoresis of 16 S rRNA PCR amplification.

(EtBr was added to the gel before electrophoresis to a final concentration of 1.5 µg/ml, followed by separation at 80 V for 40 min. The gel was exposed to UV light and the picture taken with a gel documentation system)

Lane 1 contains DNA ladder that consists of 15 ligated, evenly spaced DNA fragments ranging from 200 bp to 3000 bp at 200 bp increments. Lanes 2 to 8 contains *E. coli* isolates treated with different concentration of tuibur and nicotine: Lane 3 (100% tuibur) and lane 7 (0.01% nicotine) isolates were showed single band in 1.5% agarose gel. The amplicon sizes were near to 1500 bp (Fig.8).

The partial amplified PCR product of 16S rRNA gene was sequenced for 100% tuibur treated, Nicotine (Positive control) treated and untreated (control) *E. Coli*. In multiple sequence alignment, the control, tuibur, and nicotine treated *E. coli* stain DH5α sequences were aligned. There are many matching identical nucleotide base pairs and also substitution mutations (transition and transversion) and indel mutations (insertion and deletion) (Fig.9). All the variations between 100% tuibur treated, Nicotine (Positive control) treated and untreated (control) *E. Coli* are listed in Table.1.

Sequence analysis of 16s rRNA:

```
E. Coli DH5α CLUSTAL O(1.2.4) multiple sequence alignment

100             CGGATGAGTGGCGAACGGGTGAGTAACACGTGGGAAATCTGCCTATAAGTGGGGGATAAC   60
control         CTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAA--CTGCCTGATGGAGGGGGATAAC   58
NICOTINE        CTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAA-CTGCCTGATGGAGGGGGATAAC   59
                * ** ******** ************    ******  ******    * **********

100             ATTCGGAAACGGATGCTAATACCGCATATATTCTTGGACCGCATGGTCCTTGATGAAGAC   120
control         TAC-TGGAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGG   117
NICOTINE        TACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGG   119
                     * *****  **************   **       *  * *           *

100             GGCTTTGCTGTCACTTATAGATGATCCCGCGGCGTATAGTTAGTTGGTG--GGGTAATGG   178
control         CCTCTTGCCATCG-----GATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGGTAACGGC   172
NICOTINE        CCTCTTGCCATCG-----GATGTGCCCAGATGGGATTAGCTAGTAGGTGGG-GTAACGGC   173
                    ****  **             ** *  * *  *** **** ****   *  *  *

100             CCTACCAGACGATGATACGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGA   238
control         TCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCASGCACACTGAACTGAGAC   232
NICOTINE        TCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGA   233
                 *  * ** *** *** * **** *  ******** *** *   *******

100             CACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGATCTTCCACAATGG     287
control         A--CGGTCAGACTCCTACGGGAGGCAGCAGTGGGGATTTTGCACATGGG     279
NICOTINE        CACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATG     282
                    *  ************************ ****   *       *
```

Fig. 9. Multiple Sequencing Alignment of *E. coli* Strain DH5α;

(Control, treated with tuibur 100% and Nicotine 0.01%)

Control	Nicotine	100%
T	T	**G**
C	C	**T**
G	G	**A**
T	T	**C**
G	G	**A**
T	T	**C**
C	C	**G**
-	**A**	**A**
-	-	**T**
G	G	**A**
A	A	**T**
T	T	**A**
G	G	**A**
A	A	**T**
T	T	**A**
A	A	**T**
C	C	**T**
-	**T**	**C**
T	**G**	**G**
G	**A**	**A**
T	T	**A**
A	A	**T**
A	A	**T**
C	C	**A**

i

Control	Nicotine	100%
G	G	**T**
G	G	**T**
C	C	**T**
A	A	**G**
A	A	**G**
G	G	**A**
A	A	**C**
C	C	**G**
A	A	**C**
A	A	**T**
A	A	**G**
G	G	**T**
G	G	**C**
G	G	**C**
G	G	**T**
G	G	**T**
A	A	**G**
C	C	**A**
C	C	**T**
T	T	**G**
T	T	**A**
T	T	**A**
C	C	**A**
G	G	**A**

ii

Control	Nicotine	100%
G	G	**C**
C	C	**G**
C	C	**G**
T	T	**C**
C	C	**T**
C	C	**T**
A	A	**G**
G	G	**A**
-	-	**CTTAT**
G	G	**A**
A	A	**G**
T	T	**A**
G	G	**T**
T	T	**G**
G	G	**A**
C	C	**T**
A	A	**C**
A	A	**C**
T	T	**G**
G	G	**C**
A	A	**T**
T	T	**A**
C	C	**T**
A	A	**T**
GG	GG	**-**
G	**-**	**G**
T	T	**G**
A	A	**T**
C	C	**A**
G	G	**T**
C	C	**G**
T	T	**C**
A	A	**T**
C	C	**A**
T	T	**C**
G	G	**A**
C	C	**T**

iii

Control	Nicotine	100%
C	C	**A**
C	C	**G**
T	T	**C**
G	G	**A**
T	T	**C**
A	A	**G**
C	C	**T**
A	A	**G**
A	**C**	**C**
C	**A**	**A**
A	**C**	**C**
C	**A**	**A**
T	**C**	**C**
G	**T**	**T**
A	**G**	**G**
A	**G**	**G**
C	**A**	**G**
T	**A**	**A**
G	**C**	**C**
A	**T**	**T**
C	**G**	**G**
-	**CAC**	**CAC**
C	**G**	**G**
G	**T**	**C**
T	**C**	**C**
G	G	**A**
T	**A**	T
T	T	**C**
T	**A**	T
G	**T**	**C**
C	**G**	C
A	**C**	A
C	**A**	C
A	**C**	A
T	**A**	**A**
G	**A**	**T**
G	**T**	G

iv

Table. 1 (i, ii, iii, iv). List of mutations found in partial 16s rRNA gene

Control (Blank), Tuibur (100%) and nicotine (0.01%) treated *E. coli.*

Tuibur treatment mostly induced more substitution mutations (transition and transversion) in *E. coli* compared with nicotine treatment (Table 1).

Tuibur	
Substitution	**107**
Transition	43
Transversion	64
Indels	**7**
Insertion	5
Deletion	2
Total	**114**

Nicotine	
Substitution	**28**
Transition	6
Transversion	22
Indels	**4**
Insertion	3
Deletion	1
Total	**32**

(A)

Table.2. Mutation profiling in tuibur (A) and nicotine (B) treated *E. coli*

Higher frequency mutation has been found in 100% tuibur treated *E. coli* compared to nicotine treated. The rate of transversion was higher compared to transition mutation. Fourteen substitutions and 3 indels were found commonly in both samples. Transition: transversion (Ti: Tv) ratio was higher in 100% tuibur treated *E. coli* (67.2%) compared with Nicotine treated *E. coli* (27.3%) (Table 2).

Control and nicotine treated *E. coli* were having more similarity showing their genetic closeness, but 100% tuibur treated *E. coli* was more dissimilar (Fig. 10). The evolutionary divergence between the control and nicotine treated *E. coli* sequence is 0.0109 as well as evolutionary divergence between the 100% tuibur treated *E. coli* and control is 0.4828 and with nicotine is 0.4664 (Table 3).

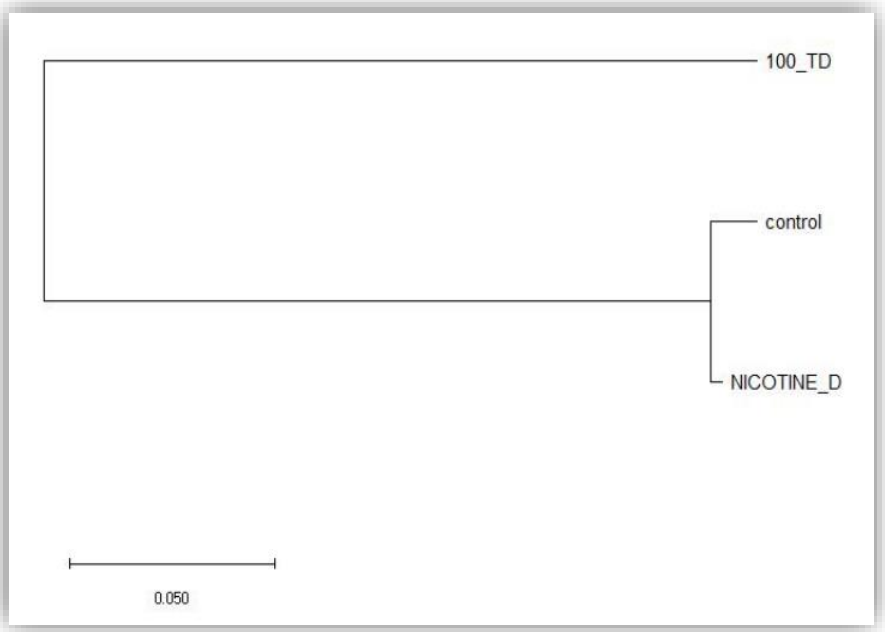

Fig. 10. Molecular Phylogenetic analysis by Maximum Likelihood method

(The evolutionary history was inferred by using the ML method based on the Tamura-Nei model (Tamura *et al.,* 1993). The tree with the highest log likelihood (-659.20) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 3 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There was a total of 292 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al.,* 2018).

	100% Tuibur	**Control**	**0.01% Nicotine**
100%			
Control	0.4828		
0.01% Nicotine	0.4664	0.0109	

Table.3. Estimates of Evolutionary Divergence between Sequences

(The number of base substitutions per site from between sequences is shown. Analyses were conducted using the Maximum Composite Likelihood model (Tamura *et al.,* 2004). The analysis involved 3 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There was a total of 292 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al.,* 2018).

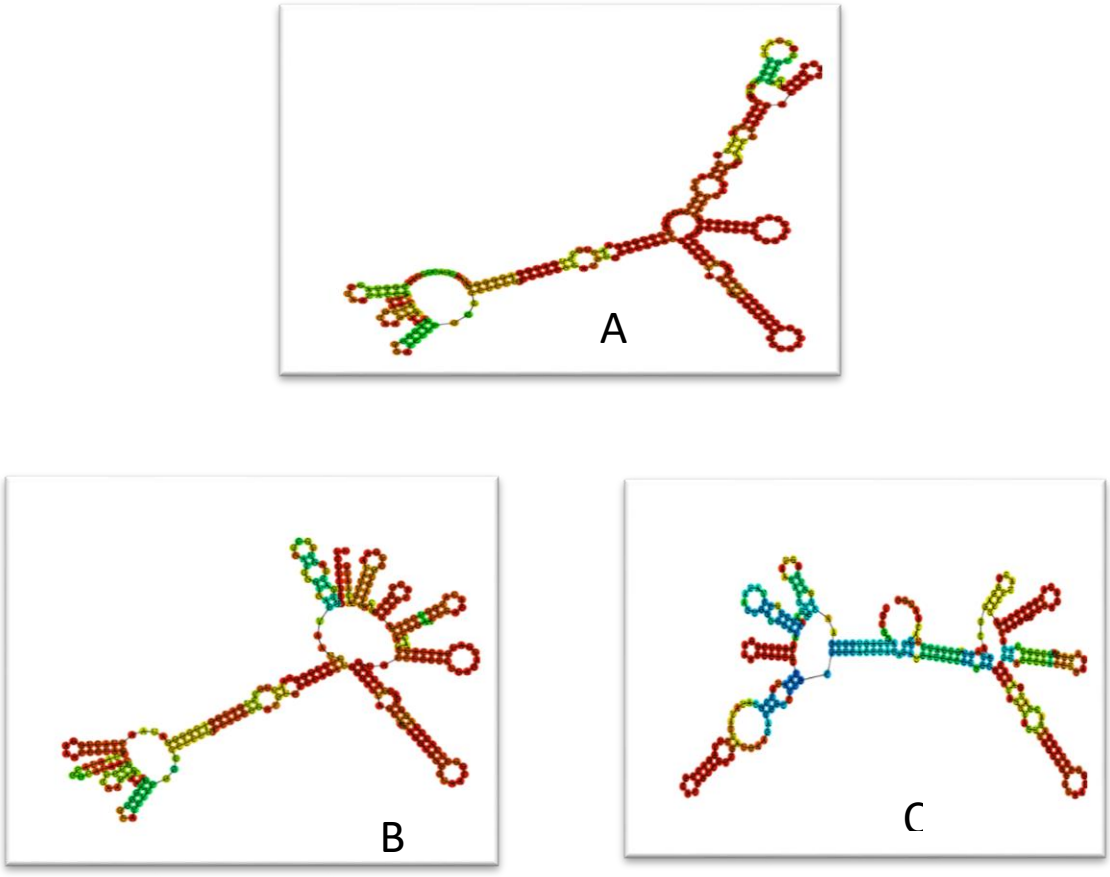

Fig. 11. Comparison of the secondary structure of 16s rRNA gene among control (A), Nicotine (B), and 100% tuibur (C) treated *E. coli.*

In this study, using RNAfold web server (in silico tool), the stem-loop structure of 16S rRNA was compared along with its three-dimensional conformation. The existence of various mutations in 100 % tuibur and nicotine (positive control) treated *E. coli* of 16S rRNA shows alteration in the stem-loop structure when compared with untreated *E. coli* (Fig. 11).

Discussion

The total amount of biomass was significantly decreased with the treatment of 100% tuibur and nicotine in *E. coli*. Tobacco can generate ROS which induces cell damage (Lalruatfela *et al.*, 2017) and specially tuibur contains heavy metals and an enormous amount of nicotine, quinoline, and hydroxyquinoline, and these compounds can damage the cell (Zhao *et al.*, 2014). This might be the cause for a significant decrease in the amount of biomass compared to control in *E. coli*. In this study, it has been found that at the third week of treatment, biomass yield has decreased drastically, which shows that the effect of stress-induced by tobacco was highest during that treatment period.

In this study, the total protein was gradually decreasing with a higher amount of tuibur and nicotine, and the change was significant in 40% tuibur treated *E. coli* which shows that oxidative stress produced by tuibur and nicotine through free radicals (ROS), decreases the protein amount in *E. coli* (Zhao *et al.,* 2014).

Catalase activity was reduced with a gradually higher concentration of Tuibur and nicotine, which shows that tobacco might have produced cytotoxic and mutagenic effects due to the reported contents of tobacco in free radicals and its ability to produce oxyradicals during microsomal electron transport and free radicals inhibits the catalase activity (Mendez-Alvarez *et al.,* 1998), which supports the result of the present study. Catalase (CAT) functions mainly to remove hydrogen peroxide formed during the metabolic stress induction in bacteria. An increase in CAT activity is supposed to be an adaptive trait possibly to overcome the damage to the cell metabolism, by reducing toxic levels of hydrogen peroxide produced during cell metabolism. Catalase activity changes have been studied in many other bacteria. CAT activity has been found to increase in the nicotine-degrading bacterium *Pseudomonas sp*. HF-1, *Bacillus substilis* and *E. coli* K12 (Shao *et al.*,2009).

The enzyme is important for the maintenance of reduced form of the glutathione in the cell at high levels because reduced glutathione is itself a free radical scavenger. Elevated levels of GR activity could increase the ratio of NADP+/

NADPH thereby ensuring the availability of NADP+ to accept electrons from the electron transport chain, and minimizing the reduction of oxygen and formation of superoxide radicals. Glutathione activity drastically decreased in this study. Nicotine or tobacco and Tuibur can produce ROS which can induce higher oxidative stress in the culture and reduce glutathione level in cells and can inhibit catalase and glutathione activity (Aspera-werz *et al.,* 2018).

In this study, SOD activity was significantly increasing day by day with the higher concentration of nicotine and tuibur compared to blank. Generally, nicotine or tobacco use to produce oxidative stress in *E. coli* and other bacteria as well. SOD is the first defence line against oxidative stress and might be the expression of SOD and DNA repair genes can inhibit DNA damage under oxidative stress (Kim *et al.,* 2004). So, there was a significant increase in SOD activity to protect the bacteria against the oxidative stress induced by tuibur and nicotine in this study. It has been reported that different bacteria possessed distinct SOD isoforms to cope with oxidative stress caused by nicotine (Shao *et al.,* 2009).

In phylogenetic analysis, nicotine treated and Control sequences were more similar, while 100% tuibur treated was showing more polymorphisms or mutations. 100% tuibur treated was having more mutations than nicotine which supports the result of phylogenetic data. The result of the present study is showing that 100% tuibur having more effect on *E. coli* at gene level compared to nicotine.

Nicotine exposer is not enough to increase the ROS level alone (Aspera-werz *et al.,* 2018), while tuibur contains lots of aromatic compounds that can generate bacterial cell death by inducing ROS. So, this might be a cause of finding a higher mutation in tuibur treated (high dose) *E. coli*. In this study, variations might occur due to tuibur stress which results in the accumulation of substitutions in 16S rRNA genes leading to the evolutionary changes (Kuo *et al.,* 2009).

This study indicates that alteration in the stem-loop structure plays a crucial role in the proper functioning of 16S rRNA in a tuibur environment. The various mutation in the sequences of treated 16S rRNA might allow them to function well in the tuibur environment by changing the folding pattern of ribosomal RNA. Further In

vitro and clinical studies must be conducted in the future to confirm our in-silico observations. The sequences in the stem-loop part of 16S ribosomal RNA (rRNA) are crucial for predicting stress and resist antibiotics. The prediction of three-dimensional structure helps to understand the role of a specific mutation in 16S rRNA (Singh *et al.,* 2018).

The present study is the first major scientific endeavor in elucidating the effects of tuibur on *E. coli*. In this study, we identified 16S rRNA gene mutation and changes in cellular antioxidant enzymes Such as Catalase, SOD, and GR. The multiple xenobiotic chemicals present in tobacco smoke derived into tuibur solution leads to many mutations and indels in 16S rRNA and their folding pattern. This also induces more biochemical stress to *E. Coli* cells. Alterations in the activity of antioxidant enzymes (CAT, SOD, and GR) were observed which might help the bacteria to survive in a tuibur containing environment.

SUMMARY

- The total Biomass and protein contents in *E. coli* treated with nicotine and tuibur decreased which might be due to the Oxidative stress.

- Nicotine (tobacco) and Tuibur treatments reduced the catalase and glutathione reductase activities in a dose dependent manner.

- SOD is the first defence line against oxidative stress which showed significant increase in activity with the higher concentration of tuibur treatment.

- In phylogenetic analysis, tuibur treatment lead to mutations in the 16S rRNA gene of *E. coli*.

- The alteration in the stem-loop structure plays a crucial role in the proper functioning of 16S rRNA in a tuibur environment by changing the folding pattern of ribosomal RNA.

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ABBREVIATIONS

Abbreviations	Full Form
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CAT	Catalase
GR	Glutathione Reductase
ML	Maximum likelihood
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
OD	Optical density
SOD	Superoxide Dismutase
rRNA	Ribosomal ribonucleic acid
TAE	Tris acetate EDTA

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DEPARTMENT	: BIOTECHNOLOGY
TITLE OF DISSERTATION	: Tuibur stress-induced antioxidant enzyme and mutation profiling in bacteria
DATE OF ADMISSION	: 06.08.2019
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1. DRC	: 28.05.2020
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ABSTRACT

TUIBUR STRESS-INDUCED ANTIOXIDANT ENZYME AND MUTATION PROFILING IN BACTERIA

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY

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DEPARTMENT OF BIOTECHNOLOGY

SCHOOL OF LIFE SCIENCES

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INTRODUCTION

Tobacco is produced from a genus of herbs *Nicotiana* and is used in many forms, including smoke and smokeless. In India, a wide range of smokeless tobacco products are used (Bhonsle *et al.,* 1992). The alkaloid nicotine, a secondary metabolite, is the main active component of tobacco and is responsible for its drugs and calming properties. An undescribed tobacco product called "tuibur" was found to be used in Mizoram during the Global Youth Tobacco Survey (Sinha *et al.,* 2004). The smokeless tobacco product, which is a unique aqueous concentrate of tobacco smoke is known locally as tuibur. A cultural tradition has been the use of tobacco in this particular form. The use of Tuibur is prevalent in both smokers and non-smokers and may be one of the significant reasons for the high prevalence of stomach cancer among the population in Mizoram (Phukan *et al.,* 2005; Mukherjee *et al.,* 2020; Chakraborty *et al.,* 2021).

Tobacco is known to contain different types of chemical species (hydrophilic and hydrophobic), many of which have been reported to have carcinogenic and cytotoxic properties (Yadav *et al.,* 2018). Heavy metals like arsenic, cadmium, chromium, lead, nickel, etc. present in tobacco have also been found to cause glomerular dysfunction (Lalruatfela *et al.,* 2017). Some of the common carcinogenic chemicals found in tobacco include quinoline, nitrite, cadmium, lead, arsenic, nickel, chromium, hydrazine, benzo(a)pyrene, N' - nitrosonornicotine, N' - nitrosoanatabine, N'-nitrosoanabasine, 4-(methylnitrosamino)-1-(3-pyridyl)-1- butanone, N-nitrosodimethylamine, etc. (Talhout *et al.,* 2011). Tuibur also contains other trace heavy metals from tuibur using ICP-MS such as Cd, Zinc, Pb, Ni, As, Cu, Cr, and Mn in tuibur (Lalmuanpuii, 2016; Muthukumaran, 2016).

In previous studies, it was also suggested that cadmium could also accentuate the production of ROS (Manca *et al.,* 1994; Bagchi *et al.,* 1997; Liu and Jan, 2000) which in turn causes elevation of lipid peroxidation, ultimately results in DNA damage (Liu *et al.,* 2009). An oxidative imbalance imposed by ROS in tissues and cellular components

causing damage to membranes, DNA, and proteins is involved in the process of lead-induced oxidative stress (Patra *et al.*, 2011)

In between Gram-negative and Gram-positive strains, the levels of antioxidant enzymes differ markedly. Gram-positive strains do not respond to exposure to nicotine, while Gram-negative bacteria *E. coli* K12 and *Pseudomonas* sp. HF-1 showed higher nicotine stress sensitivity. Thus, Gram-negative bacteria are the more suitable organisms for the studies on environmental nicotine pollution (Yadwad *et al.*, 1990).

In prokaryotic cells, during the metabolism of nicotine, reactive metabolites were released, that are not appropriately inactivated, may eventually form a covalent (irreversible) bond to cellular DNA leading to the formation of deleterious DNA adducts (Shao *et al.,* 2009). The 16S rRNA gene sequencing is used as a tool to identify bacteria at the species level and assist with differentiating between closely related bacterial species. The most common primer pair was designed by Weisburg *et al.* (1991) and is currently referred to as 27F and 1492R; however, for some applications, shorter amplicons may be necessary. In addition to highly conserved primer binding sites, 16S rRNA gene sequences contain hypervariable regions that can provide species-specific signature sequences useful for the identification of bacteria.

The purpose of this study is to provide data on the antioxidant enzymes such as CAT, GR, SOD, and genetic alterations in DH5α *E. coli*, a gram-negative bacterium, treated with tuibur.

MATERIALS AND METHODS

In this study, *Escherichia coli* strain DH5α (Gram-negative bacteria) was cultured at 37ºC at 120 rpm in liquid LB medium (Ausubel *et al.,* 1999). Bacterial cultures were sub-cultured to new LB broth after 24 h and kept for 1 h incubation at 37ºC in 120 rpm and after 1 h of incubation tuibur treatment (different concentration;

i.e., 10%, 40%, 100%, and 0.01% nicotine) were performed daily for 30 days during sub-culturing. The optical density was measured after 24 h at 600 nm. The bacterial cells were pelleted by centrifuging at 4000 rpm for 10 min at 4 ºC and the biomass of growth, total protein, and antioxidant enzymes were estimated every once a week for a month during the treatment schedule.

To scavenge reactive oxygen species, cells were equipped with a very efficient antioxidative defence system that protects them from the destructive oxidative burst. Oxidative burst under stress was assessed by studying protein content and antioxidative enzyme activities viz. CAT, GR, and SOD in *E. coli* using UV-Visible Spectrophotometer.

Bradford method was used to estimate total protein. Absorbance will increase over time; samples were incubated at room temperature for 1 h and absorbance was measured at 595 nm (Bradford, 1976).

The activity of catalase was determined according to the method of Aebi (1983). Catalase activity can be measured by following either the decomposition of hydrogen peroxide or the liberation of oxygen. The UV spectrophotometric technique is the method of choice for biological material. A decrease in extinction per unit time at 240 nm can be directly followed by the decomposition of Hydrogen peroxide. A catalase activity measure is a difference in extinction per unit time.

Glutathione reductase catalyzes the reduction of glutathione disulfide (GSSG) involving the oxidation of NADPH. Catalytic activity is measured by following the decrease in absorbance due to the oxidation of NADPH. GR activity was calculated by measuring the oxidation of NADPH at 340 nm (Habig *et al.*, 1974).

The assay of SOD is based on the inhibition of the rate of reduction of cytochrome C by the superoxide radicals. In a spectrophotometer, the readings were registered every 20 seconds at 340 nm for a minimum of 4 minutes (Singh, 2007).

SPSS 22, GraphPad-9, R kit, and Microsoft Excel 2013 were used to conduct the statistical analysis.

Overnight cultures of the tuibur treated bacterial strain were grown in liquid LB medium. The DNA was extracted by maxiprep phenol extraction and ethanol precipitation method (Ausubel *et al.*, 1999).

The DNA regions containing the 16S rRNA gene was PCR amplified using oligonucleotide primers. The isolates were subjected to amplification of the 16S rRNA gene by using universal primers [forward 16S primer (27F)- 5' AGAGTTTGATCMTGGCTCAG 3' and reverse primer (1492R) – 5' GGTTACCTTGTTACGACTT 3'] (Heuer *et al.,* 1997). The PCR bands were analyzed documented using Gel documentation system (BioRAD, USA) and were purified by the Pure Link PCR purification kit, and sequenced by the Sanger methodology with the Big Dye Terminator v 3.1 Automated Cycle Sequencing using ABI 3500 instrument according to manufacturer protocol (Thermo Scientific USA). All the sequencing data were edited with Geospiza's FinchTV 1.4 (Treves, 2010) and are analyzed by BLAST, MEGA X, Clustal Omega (Kimura, 1980; Tamura *et al.,* 2004; Kumar *et al.,* 2018). 16S rRNA secondary structure was predicted from DNA sequences of 16S rRNA using an RNAfold web server. In this study, Using the RNAfold web server **(**in silico tool), the stem-loop structure of 16S rRNA along with its three-dimensional conformation was analyzed (Singh *et al.,* 2018).

RESULT

The *E. coli* cells were treated with different concentrations (10%, 40%, and 100%) of tuibur and Nicotine (0.01%) as a positive control for 24 h daily for 29 days in a continuous manner. We observed relatively higher biomass in Blank and 10% tuibur, relatively moderate biomass in 40% tuibur, and Low biomass on 100% tuibur and positive control samples throughout the study period. But, the biomass of all the samples

increased at end of the study. Overall estimation of biomass yield culture treated with 100% tuibur (p=0.01) and nicotine (p=0.03) showed significance when compared with the control (Fig.2).

We observed a relatively higher amount of total protein in Blank and 10% tuibur, a relatively low amount of total protein in 40% tuibur, and a moderate amount of total protein in 100% tuibur as well as positive control samples throughout the study period. Overall estimation of total protein compared with the control; culture treated with 40% tuibur (p=0.01) showed significance.

We observed a dose-dependent manner of catalase activity in the initial period of treatment. On day 29, the catalase activity showed the most significant value compared with the other days.

The activity of glutathione reductase gradually decreased on day 8 and after that, it decreased drastically. Compared to the days 15 and 29, the day 1, 8 and 22 showed significance.

SOD activity decreased steadily during the study period. Compared to day 29, days 1, 8, and 22 were significant. High significance was seen on day 1^{st}, $8^{th,}$ and 15^{th}.

The partial amplified PCR product of the 16S rRNA gene was sequenced for 100% tuibur treated, Nicotine (Positive control) treated, and untreated (control) *E. Coli*. Tuibur treatment mostly induced more substitution mutations (transition and transversion) in *E. coli* compared with nicotine treatment. Transition: transversion (Ti: Tv) ratio was higher in 100% tuibur treated *E. coli* (67.2%) compared with Nicotine treated *E. coli* (27.3%). Control and nicotine treated *E. coli* were having more similarity showing their genetic closeness, but 100% tuibur treated *E. coli* was more dissimilar. The evolutionary divergence between the control and nicotine treated *E. coli* sequence is 0.0109 as well as evolutionary divergence between the 100% tuibur treated *E. coli* and control is 0.4828 and with nicotine is 0.4664.

In this study, using RNAfold web server (in silico tool), the stem-loop structure of 16S rRNA was compared along with its three-dimensional conformation. The existence of various mutations in 100 % tuibur and nicotine (positive control) treated *E. coli* of 16S rRNA shows alteration in the stem-loop structure when compared with untreated *E. coli.*

CONCLUSION

The total Biomass and protein contents in *E. coli* treated with nicotine and tuibur decreased which might be due to Oxidative stress. Nicotine (tobacco) and Tuibur treatments reduced the catalase and glutathione reductase activities in a dose-dependent manner. SOD is the first defence line against oxidative stress which showed a significant increase in activity with the higher concentration of tuibur treatment. In phylogenetic analysis, tuibur treatment led to mutations in the 16S rRNA gene of *E. coli*. The alteration in the stem-loop structure plays a crucial role in the proper functioning of 16S rRNA in a tuibur environment by changing the folding pattern of ribosomal RNA.

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