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Evaluation of genotoxicity caused by the carcinogenic benzo[a]pyrene, a common ingredient of Indian tobacco chew, using a bacterial gene expression model.

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Tobacco consumption (chewing and smoking) mainly causes many forms of cancer, such as Lung cancer, kidney cancer, cancer of the larynx, head, neck, bladder, esophagus, pancreas, liver etc. Tobacco products contain various carcinogens such as Polycyclic aromatic hydrocarbons (PAHs), *N*-nitrosamines, aromatic amines, 1,3-butadiene, benzene, aldehydes, and ethylene oxide are the most important carcinogens. Cytochrome P450 enzymes are important in metabolizing PAHs to epoxide intermediates, which are further converted to diol epoxides, the ultimate carcinogens. CYP-1A1/1B1 enzymes catalyze activation of pro-carcinogenic and metabolic activation of PAH. CYP-1A1/1B1 gene expression is induced by PAH and polyhalogenated hydrocarbons through aryl-hydrocarbon receptors. In this study, we used bacterial gene expression as a model system to demonstrate the lethal effects of B[*a*]P. Our results show that the gene expression in bacteria is indeed affected by B[*a*]P. Based on our results, we extrapolate that B[*a*]P can cause cancer by changing the gene expression levels in humans in a similar way that was observed in this study.

Keywords: Tobacco chew, B[a]P, Carcinogen, cyp450, Cancer, PAH, smoking.

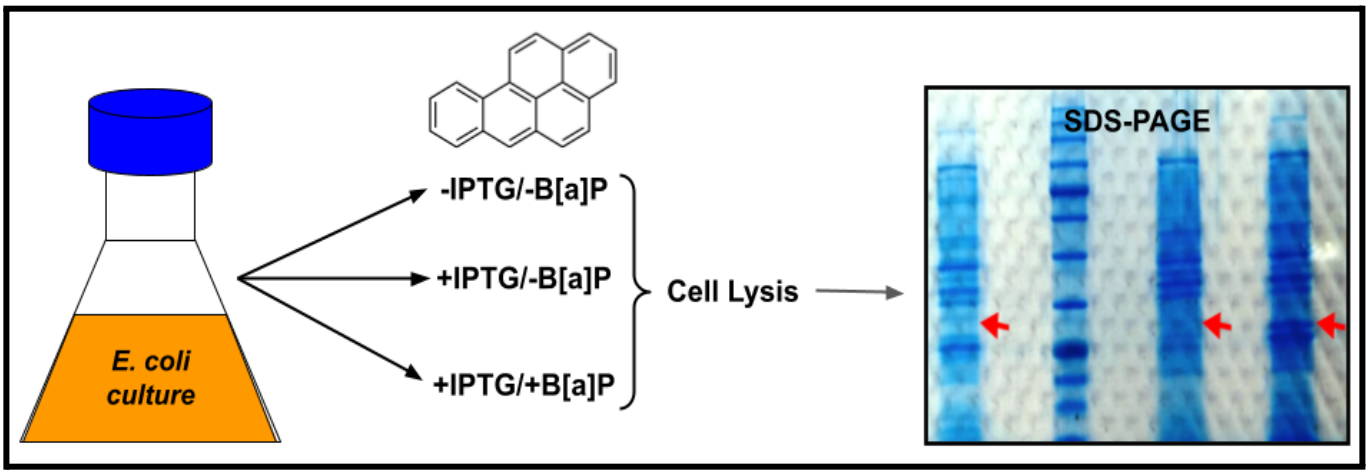

Figure 1. Overview of bacterial gene expression analysis in the presence and absence of B[a]P followed by SDS-PAGE analysis.

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The condition in which cells divide uncontrollably and spread into surrounding tissues called CANCER. There are more than 100 types of cancers known (according to the organ or tissue affected). There are various types of cancer; Carcinoma, Sarcoma, and Leukemia. Proto-oncogenes, tumor suppressor genes, and DNA repair genes are responsible for genetic changes in the cell, which leads to cancer. Sometimes, these genetic changes are called “drivers" of cancer. Proto-oncogenes participate in normal cell growth and division. Tumor suppressor genes also control cell growth and division. DNA repair genes participate in fixing damaged DNA.Additional mutations and changes in their chromosomes develop in these genes of a cell, like duplications and deletions of chromosome parts. *Carcinoma* is a cancer of the epithelial cell, which covers the surfaces of the body. *Sarcomas* are the type of cancers formed in many tissues like soft and fibrous tissues, bones and muscle, fat, blood vessels, lymph vessels etc. Cancers that form in the blood are called *leukemias.* These cancers are not solid tumors. Instead, large numbers of abnormal leukocytes increased in the blood and bone marrow, with decreased normal blood cells.

According to the National Cancer Institute, our lifestyle choices are known to increase your risk of cancer. Women should not drink or smoke more than one time per day and men should not drink or smoke twice per day,frequent exposure to sun or continuous blistering sunburns, obesity, and unsafe sex can cause cancer. People who smoke or chew tobacco products like cigarettes and tobacco chew mainly cause lung cancer and mouth cancer. LUNG CANCER: Lung cancer is a type of cancer that forms in the lungs. Globally, lung cancer is the main cause of cancer deaths. Smokers have the greatest risk of lung cancer, even non-smokers can also cause lung cancer. The risk of lung cancer increases with the number of cigarettes we've smoked in a particular period of time. If we quit smoking, even after smoking for many years, we can reduce your chances of developing lung cancer. In the earliest stages, lung cancer doesn't show any signs and symptoms. Symptoms of lung cancer are seen when the disease is advanced. Signs and symptoms of lung cancer: Non-stop cough, Coughing up blood, in small amounts, Shortness of breath, Chest pain, Hoarseness, Losing weight, Bone pain, Headache.

Smoking causes mainly lung cancers - both in active and passive smokers. Doctors believe smoking damages the cells that line the lungs and causes lung cancer. Inhaling cigarette smoke immediately changes the lung tissue. Initially our body may be able to repair this damage. After each repeated exposure, normal cells lining our lungs are increasingly damaged. Types of lung cancer: Based on the observation of lung cancer cells under the microscope, doctors divide lung cancer into two major types. Based on the type of lung cancer, treatment is decided. The two types of lung cancer: Small cell lung cancer: Small cell lung cancer is less prevalent than non-small cell lung cancer and nearly exclusively affects heavy smokers. Non-small cell lung cancer: Non-small cell lung cancer (NSCLC) is a catch-all name for a variety of lung malignancies.Non-small cell lung cancers include squamous cell carcinoma, adenocarcinoma, and large cell carcinoma.

Tobacco refers to a group of plants of the *Solanaceae* family's *Nicotiana* genus, as well as any product derived from their cured leaves. Tobacco comes in over 70 different species, although *N. tabacum* is the most prevalent commercial crop. In some areas, the more potent variety *N. rustica* is also used. Tobacco includes both nicotine and harmala alkaloids, which are extremely addictive stimulants. Tobacco use is linked to a variety of severe diseases, including those affecting the heart, liver, and lungs, as well as a variety of malignancies. Tobacco use was rated the world's single largest avoidable cause of death by the World Health Organization in 2008. Tobacco in the Americas has been reported back to 1400-1000 BC in Mexico. Tobacco has long been grown and used by many native Americans.

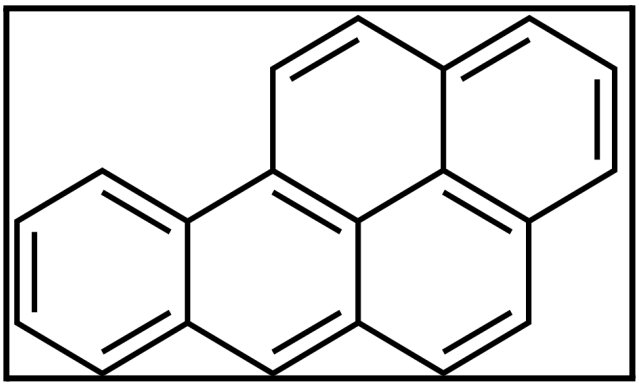

Figure 2. Two-dimensional structure of Benzo[a]Pyrene.

Tobacco was traditionally carried in pouches by people from the Northeast Woodlands tribes as a commonly acceptable trade item. It was smoked for social purposes as well as for ceremonial purposes, such as to seal a peace treaty or a trade transaction. Tobacco is seen as a gift from the Creator in several Native tribes, with ceremonial tobacco smoke sending one's thoughts and prayers to the Creator. The Tobacco Board of India is headquartered in Guntur, Andhra Pradesh. There are 96,865 tobacco farmers registered in India, with many more unregistered. In 2010, there were 3,120 tobacco product production plants in India. Tobacco production takes up about 0.25 percent of India's cultivated land. The tobacco business has been sponsored by the Indian government since 1947. Tamil Nadu, Andhra Pradesh, Punjab, Bihar, Mysore, and West Bengal each have a tobacco research center, with the central research institute in West Bengal. In genetics, several tobacco plants have been employed as model organisms. Tobacco BY-2 cells, which are generated from the *N. tabacum* cultivar 'Bright Yellow-2,' are one of the most important plant cytology research tools. Tobacco has laid the framework for modern agricultural biotechnology by pioneering callus culture research and elucidating the mechanism through which kinetin functions. In 1982, *Agrobacterium tumefaciens* was used to make the first genetically engineered plant, an antibiotic-resistant tobacco plant. This study paved the way for all genetically engineered crops to follow.

CARCINOGENIC METABOLITES IN TOBACCO: The smoke that comes out of a cigarette's mouthpiece is an aerosol with around 1010 particles per milliliter and 4800 chemical components. A glass fiber filter has been used to separate cigarette smoke vapor components from the particle phase in experiments. The vapor phase smoke will account for more than 90% of the total smoke weight. Nitrogen, oxygen, and carbon dioxide are the most common chemicals in the vapor phase. Nitrogen oxides, isoprene, butadiene, benzene, styrene, formaldehyde, acetaldehyde, acrolein, and furan are all highly carcinogenic vapor phase chemicals. At least 3500 chemicals are found in the particulate phase of smoke, with polycyclic aromatic hydrocarbons (PAH), N-nitrosamines, aromatic amines, and metals accounting for the majority of carcinogens. In these models, PAH-containing fractions of the condensate also cause cancers, but the PAH concentrations are too low to explain the carcinogenicity. Other condensate fractions show tumor-promoting and co-carcinogenic properties, enhancing the carcinogenicity of the PAH-containing fractions. The International Agency for Research on Cancer has identified over 60 carcinogens in cigarette smoke for which there is "sufficient evidence for carcinogenicity" in either laboratory animals or people. PAH (10 compounds), aza-arenes (3), N-nitrosamines (8), aromatic amines (4), heterocyclic amines (8), aldehydes (2), volatile hydrocarbons (4), nitro compounds (3), miscellaneous organic compounds (12), metals and other inorganic compounds (12) are among the substances they include (9). Other carcinogens that have not been evaluated by the IARC are also likely to exist. Multiple alkylated and high molecular-weight compounds, for example, have been discovered among the PAHs, although their carcinogenicity has yet to be determined. PAH, aza-arenes, tobacco-specific nitrosamines, e.g. 4-(methylnitrosamino)-1-(3- pyri -dyl)-1-butanone (NNK), 1,3-butadiene, ethylene oxide, nickel, chromium, cadmium, polonium-210,

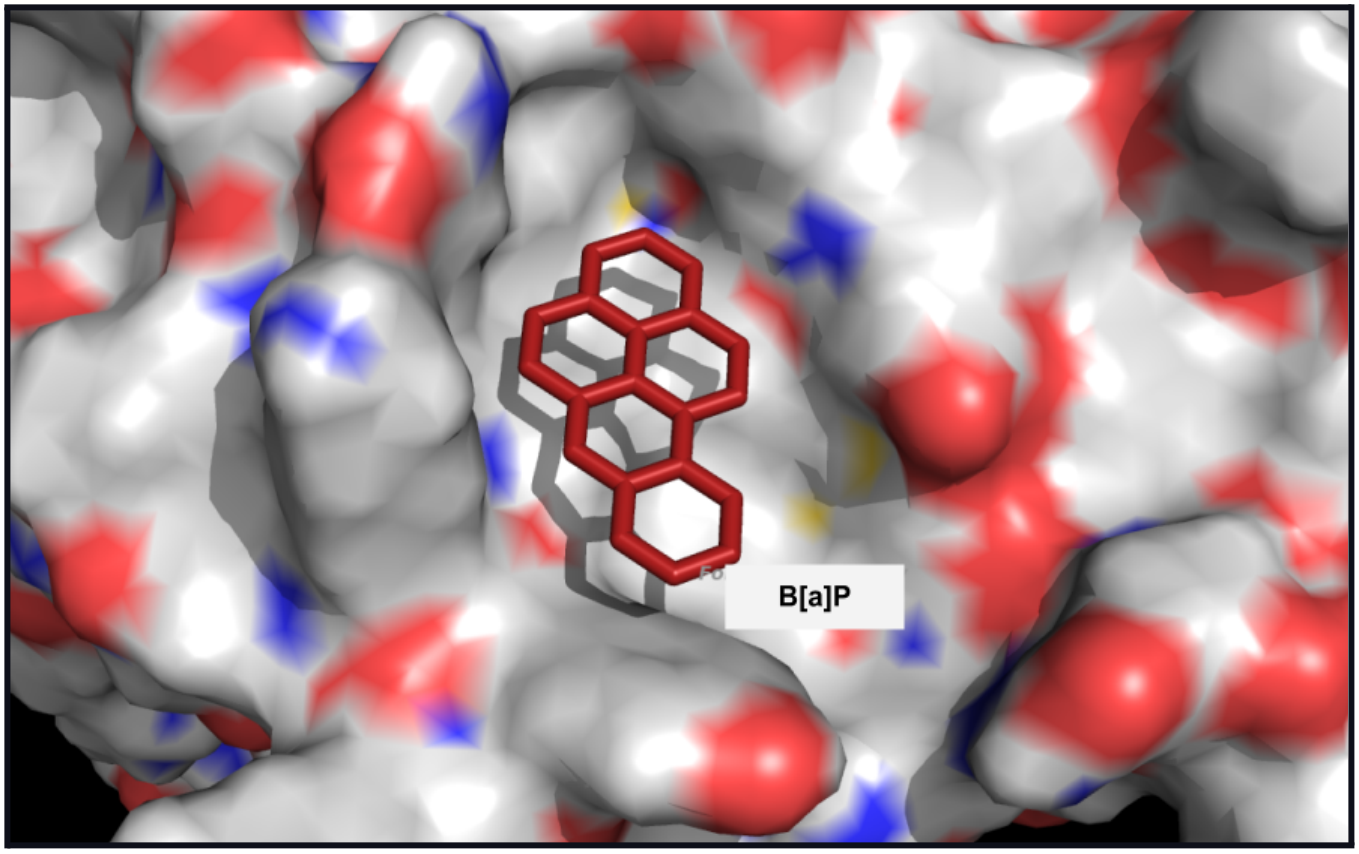

Figure 3. B[a]P docked into the active site of CYP450 1A1.

arsenic, and hydrazine are all known lung carcinogens in cigarette smoke. These chemicals have been found in cigarette smoke and have been shown to cause lung cancers in at least one animal species.

Benzo[a]pyrene (B[a]P) is the most widely investigated of the PAHs, and its capacity to cause lung tumors when administered locally or inhaled has been demonstrated conclusively. When B6C3F1 mice were given B[a]P in their diet, no lung tumors appeared. B[a]P is more carcinogenic than benzofluoranthene or indeno[1,2,3-cd]pyrene in tests of lung tumor production by implantation in rats. The existence of B[a]P in cigarette smoke has been proven by extensive analytical evidence. It has a sales-weighted concentration of roughly 9 ng per cigarette in today's 'full-flavored' cigarettes. The abundance of B[a]P literature tends to draw attention away from other topics. Benzo[a]pyrene (B[a]P) is the most thoroughly researched PAH, and its capacity to cause lung tumors when administered locally or inhaled has been demonstrated conclusively.

Cytochrome P450 enzymes catalyze the addition of an oxygen atom to a carcinogen, enhancing its solubility in water and transforming it to a more easily excretable form. Phase 2 enzymes, which convert the oxygenated carcinogen to a form that is extremely soluble in water, aid this metabolic detoxification process. The organism will be safeguarded to the extent that this process is effective. However, some of the intermediates generated by cytochrome P450 enzymes interacting with carcinogens are extremely reactive, having an electrophilic (electron-deficient) core. DNA adducts can occur when such intermediates or metabolites react with DNA.

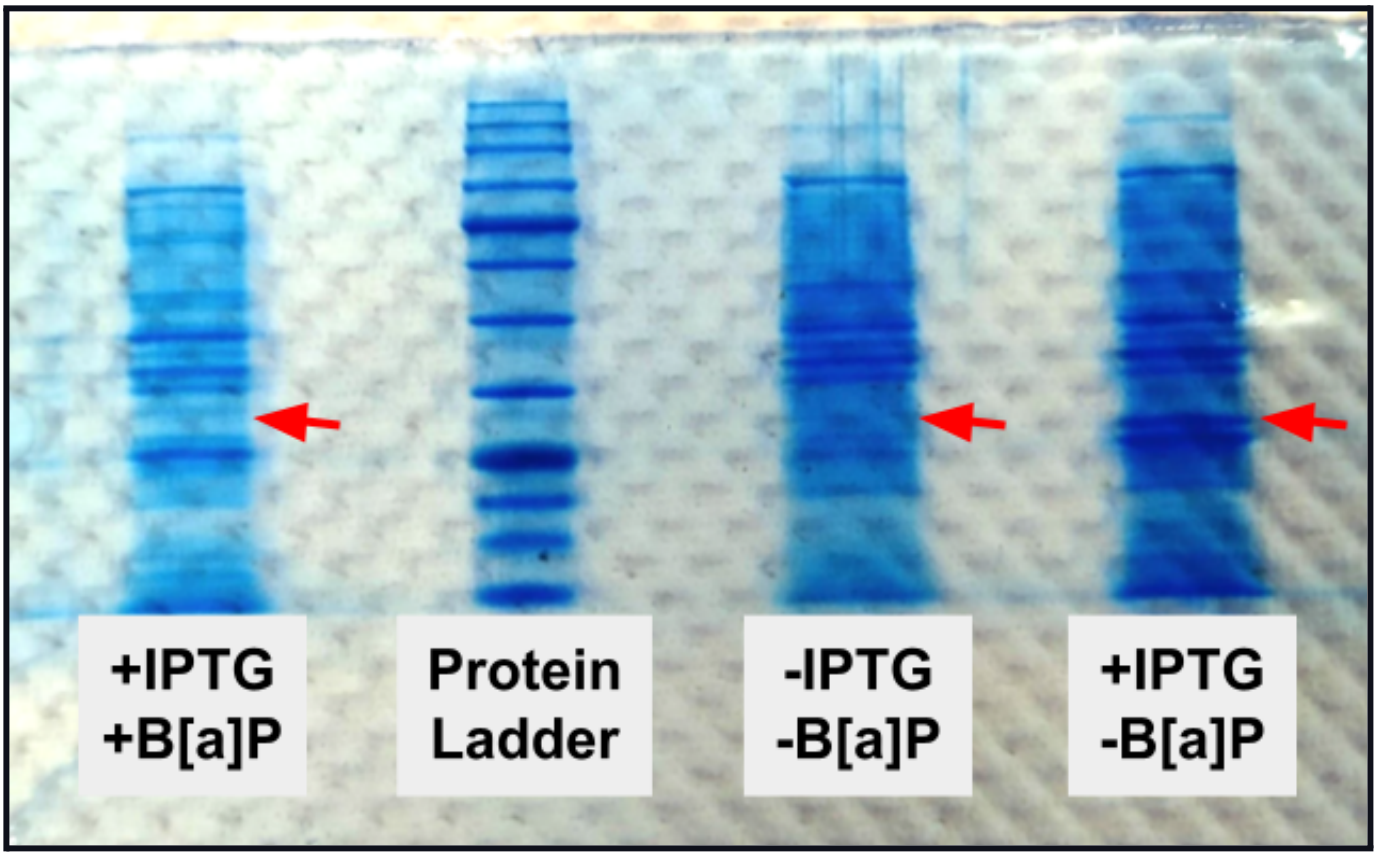

Figure 4. Gene expression analysis of cell lysates SDS-PAGE.

Metabolic activation is the process of converting an unreactive carcinogen into a form that can bind to DNA. Because DNA adducts are crucial to the carcinogenic process, the balance between metabolic activation and detoxification differs among individuals and is expected to alter cancer risk. The majority of carcinogens found in cigarette smoke require metabolic activation.To remove DNA adducts from the genome, complex DNA repair processes have emerged. P450 (CYP) 1A1 is involved in the metabolism of endogenous substrates and medicines, as well as the activation of some poisons and pollutants. CYP1A1 is well-known for its ability to biotransform polycyclic aromatic hydrocarbons into carcinogens, such as benzo[a]pyrene in tobacco smoke.

In this study, we hypothesized that B[a]P may have a direct effect on the gene expression profiles of various organs in humans thus contributing to various cancers. In order to test this hypothesis, we used a bacterial gene expression system in which we examined the protein expression levels qualitatively using SDS-PAGE. Protein expression was performed using an IPTG-inducible system in the presence and absence of B[a]P.

Materials & Methods:

Docking B[a]P into the active site of CYP450-1A1: The procedure was followed from a previously published article [21]. Briefly, the CYP450-1A1 was taken as a receptor and a docking grid was built as shown in Figure 8 into which B[a]P (ligand) was docked to obtain multiple binding poses. Exhaustive sampling was done to obtain multiple binding poses of the ligand

in order to manually verify the appropriate binding orientation using the binding affinity values as a guiding tool. The ligand 3-D structure was imported from a 2-D diagram using ChemSketcher software. The ligand was further energy minimized to avoid any steric clashes or bad contacts before docking into the docking grid built into the active site of the receptor.

Bacterial gene expression:

Preparation of LB Broth: In 100 ml of deionised water,add 2.5grams of LB broth. Autoclave it for 15minutes under 15lbs pressure.
Preparation of LB Agar: Prepare LB-Agar by adding 0.5gm of LB powder and 0.3gm of agar powder in 40ml of deionised water,(for 1 petri plate).Autoclave it for 15 minutes under 15lbs pressure.Let it cool down to luke warm.After it attains room temperature, pour it into petri dishes and let them turn into solid.
Preparation of Ampicillin (50mg/ml): Add 3 mg of ampicillin in 600 ml of deionised water. Store it at -20 ℃ until further use.

Resuspend the *E.coli* cells in vial by adding 0.25ml of LB broth. In 100 ml of deionised water, add 2.5grams of LB powder. Autoclave it for 15-20 minutes under 15lbs pressure. Prepare LB-Agar by adding 0.5gm of LB powder and 0.3gm of agar powder in 40ml of deionised water (for 1 petri plate). Add ampicillin into the LB-agar just before pouring it into the petri plates. Add Ampicillin and LB-agar into petri plates and wait until it solidifies. These plates can either be used immediately after the LB Agar solidifies or for later purposes, the plates must be stored at 4℃. After LB-agar solidifies,streak inoculum in a zig-zag pattern and incubate at 37 ℃ for 24hrs/overnight until colonies appear.

After the colonies appear, *E.coli* colonies are grown in 50ml of LB-broth and incubate it for 2hrs. Take 5 ml LB-broth in three different test tubes labeled as (1) IPTG (Isopropyl ß-D-1-thiogalactopyranoside); (2) IPTG + B[A]P and (3) control (uninduced culture) indicate as BI-Before induction (no IPTG and no B[A]P) and AI (only IPTG, no B[A]P). Incubate these tubes for 2hrs. After incubation, transfer the samples in centrifuge tubes for centrifugation. Place the samples of AI and BI in a centrifuge and run it at 8000 rpm for 10 mins. The supernatant is discarded and the pellet is retained. In these pellets, add 0.1ml of cell lysis buffer and wait for a few minutes(DO NOT VORTEX) to let lysis of the *E.coli* cells to analyze the labile macromolecules of the cells. Boil the AI and BI samples in a water bath for 8-10 mins. Centrifuge AI and BI samples at 8000 rpm for 10mins. Transfer the supernatant to a fresh tube without disturbing the pellet.
SDS-PAGE: Preparation of 12% Separating gel: 30% acrylamide-bisacrylamide -6ml, Deionised water-3ml, 2.5Xtris SDS-buffer -6ml (pH-8.8), 10%APS solution-125ul, TEMED-7.5ul. Preparation of 5% Stacking gel: 30% acrylamide-bisacrylamide-1.3ml, Deionised wat er-5.1ml, 5X tris-SDS buffer (pH-6.8)-1.6ml, 10% APS solution-7.5ul, TEMED-15ul.
SDS-PAGE has two plates .i,e., thick plate and thin plate placed vertically parallel in the plate holder. Take some distilled water and pour it in between the plates to check for any leakage. In case of any leakage, immediately change the plates. After leakage testing, discard the water and pour separating gel in between the plates filling upto half of the space. Wait until it solidifies. Add stacking gel in between the plates, above the solidified separating gel until it fills another half. Place the comb in the top of the stacking gel in between the plates in order to form wells for us to load the samples (protein) later. Wait until the gel gets solidified and remove the comb safely without disturbing the wells. Add the obtained samples of B[A]P, IPTG+B[A]P Sample, Control (no IPTG and no B[A]P) with the help of a micropipette in the well. The entire set is kept into the electrophoresis chamber which has positive and negative electrodes. With the help of a Gel running buffer, which is added inside the chamber, will

help to run entire electrophoresis for 2 hrs. The GEL, after 2 hrs, is taken out carefully from the plates without getting it torn. For staining the gel overnight, Carefully transfer the gel without tearing it, into the gel staining dye. After staining it overnight, now the gel is transferred into the destaining solution overnight so that the extra stain is removed from the gel. After the destaining process, the gel picture is taken.

Results and Discussion:

Binding pose of B[a]P in the active site of CYP-1A1: Among the nine different binding poses obtained, the best pose was selected based on its highest binding affinity as shown in Figure 3. B[a]P (shown as red color stick model) docking pose in the active site cavity of the CYP-1A1 (shown as white surface model). B[a]P being a highly hydrophobic molecule, no hydrogen bonding or electrostatic interactions were seen as expected. All interactions are of hydrophobic nature.

B[a]P decreased bacterial gene expression: Based on the SDS-PAGE analysis, it was found that the bacterial gene expression in the presence of B[a]P is relatively decreased when compared to the control (absence of B[a]P). As shown in Figure 4, the protein levels significantly decreased in the presence of B[a]P. The left most lane shows lack of protein expression in the presence of B[a]P (indicated by the red arrow). The right most lane shows presence of protein expression in the absence of B[a]P (red arrow). The two center lanes are protein molecular weight ladder (center left lane) and negative control (center right lane). The current study focused on the genotoxic effects of B[a]P on the bacterial gene/protein expression. Our studies indicate that in presence of B[a]P, the bacterial gene/protein expression decreased significantly and suggest that a similar effect is possible in humans as well, especially in people that chew and/or smoke tobacco products containing B[a]P.

B[A]P is a carcinogenic agent that causes lung cancer. The bands formed in the Gel of SDS-PAGE shows that B[A]P is affecting the normal expression of protein. Before Induction (BI), where we don't add B[A]P & IPTG no expression is seen and absence of CYP enzymes which metabolizes the Carcinogens and other drugs. After Induction (AI), where IPTG is added and B[A]P is not added to the *E.coli* culture, shows that IPTG helps the gene expression without mutation. As the structure of CYP1A1 and 1B1 with B[A]P is not available in the PDB, we constructed the structure by using the software called autodock tools. To know the exact position of binding of B[A]P to both CYP1A1 & 1B1, we will conduct X-ray diffraction to study the structure of binding of B[A]P in future and that structure can be deposited to the PDB.

Although the metabolites of B[a]P were to be blamed for carcinogenicity in humans, in this study, we did not use a humanized system yet we observed changes in gene expression suggesting that B[a]P by itself or its metabolites may cause the phenotype. Alternatively, we make an assumption that bacterial cells contain homologs of human CYP450 1A1/1A2/1B1. Deeper mechanistic studies are yet to be performed to understand the details in the future.

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Conflict of interest: This research article is an ongoing project currently at TCABS-E, Rajahmundry, India.