Applied Biochemistry and Biotechnology (2022) 194:1955–1980
https://doi.org/10.1007/s12010-022-03810-1

ORIGINAL ARTICLE

Neuropharmacological Alterations by a Rice Contaminant *Stenotrophomonas maltophilia*: a Detailed Bio-molecular and Mechanistic Landscape

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Accepted: 31 December 2021 / Published online: 10 January 2022

Abstract
Contaminated rice is a major source of food poisoning in human communities where our earlier study showed *Stenotrophomonas maltophilia*, a Gram-negative bacillus, has been a major contaminant of the stored rice. In the present study, mono- and di-unsaturated fatty acids (UFAs) such as 18:1 ω 7 c, 16:1 ω 6 c, 16:1 ω 7 c, and 18:2 ω 6,9 c long-chain fatty acids have been found as the chief constituents of *S. maltophilia* boiled cell lysate. Throughout the study, both acute and chronic exposure of the cell lysate showed a decrease in the locomotor activity and a time-dependent increase of the depression ($p < 0.001$–0.0001, two-way ANOVA), supported by bioamine (dopamine, noradrenaline, adrenaline, serotonin, and GABA) depletion in rodents' brain possibly due to UFA-amino acid decarboxylase interaction favoring bioamine depletion as revealed by our study. Furthermore, the UFA-rich cell lysate revealed dose-dependent inhibition of murine brain microglial cell viability in vitro with concomitant increase of reactive oxygen species (ROS) inside the cell. Destruction of neuroprotective and neurotrophin releasing microglial cells, augmentation of brain ROS, and inflaming brain tissue resulting in infiltration of polymorphonuclear leucocytes also suggest to cause neurotoxicity by UFA derived from *Stenotrophomonas maltophilia*.

Keywords Unsaturated fatty acid · Brain bioamines · Microglial cells · Polymorphonuclear leucocytes · Amino acid decarboxylases

Introduction

Microbial pathogenesis is a major area of pharmacology because of its serious and life-threatening invasions in various physiological systems together with organs. The dynamics of microbial infection, its propagation pathways, interaction orchestration, perturbation

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through biochemical and molecular networks of secondary messengers, and receptors have been a major focus of pharmacological research over the last few decades. Apart from toxicological manifestations, discovery of the target and route of therapeutic measures against such pathogenesis has been another primal objective of such investigations. Food borne microbial pathogenesis, its propagation dynamics, and multifaceted organic infections have been a lesser highlighted area in pharmacology.

With the tremendous onslaught of civic and urban modernization as well as population influx; proper storage, maintenance, and distribution of food are still facing challenges especially in hot, tropical, and population dense countries across the globe [1]. In tropical countries, moisture content of the grain is also responsible for microbial contamination during storage [2]. There are different strains of bacteria and fungi that not only reduce the food value of stored grains (rice) but also cause myriads of diseases and infections after entering the physiological systems. The toxins of food contaminating bacterial strains such as clostridial neurotoxins, *Staphylococcus aureus*, and *Bacillus cereus* enterotoxins cause various diseases [3]. Similarly, fungal species like *Aspergillus*, *Penicillium*, *Alternaria*, and *Fusarium* produce highly toxic secondary metabolites [4]. The microbial contaminants, even after food processing and mere cooking in household infrastructures, cause diseases like gastrointestinal tract infections, respiratory tract infections, various wounds, and skin infections [3]. The food safety, health, and food borne diseases are a worldwide concern and WHO in the year 2017 reported a loss of 33 million good health due to contaminated food [5].

The food safety and health have been an area of concern for many years with multiple works related to the contamination, evaluation of agricultural products. The microorganisms if identified causing diseases along with their pharmacological or toxicological pathways, rational treatment of diseases may be found. Based on such concept, we propose to study the pharmacological alterations generated from contaminated rice which may not get removed by the household cooking methods and subsequently alter physiological systems.

Now, in our first report regarding this study, the predominant contaminant of stored rice was identified as *Stenotrophomonas maltophilia*, a Gram-negative bacillus [6]. Rice is consumed after boiling and cooking so chances of toxicogenic candidates may be removed. To simulate the condition, the rice was boiled or hydrolyzed and introduced the boiled cell lysate inside viable physiological system for any associated alterations. Furthermore, to identify the toxicant present in the cell lysate, programmes such as Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE), PAGE coupled proteomics, GC–MS, or GC–MS-FAME (fatty acid methyl ester) analyses were undertaken. Our preliminary pilot studies revealed an altered neurobehavioral profile by this contaminating cell lysate and such altered neuropharmacological profiling of contaminating rice has not been established in any earlier report, and we have undertaken this neuropharmacological study in characteristic, quantitative, bio-molecular, and mechanistic level for further investigations.

In pursuit, the work has been elaborated in five major divisions: a general dissection of mice behavioral profile due to the effect of contaminating cell lysate, identification of the toxicants present in boiled cell lysate, molecular investigations of the cognitive changes by estimating brain neurotransmitters, observation of direct CNS damage through brain histopathology, and cytotoxicity assay of characterized cell lysate against neurotrophic and neuroprotective brain microglial cells. The earlier reports have shown the presence of this microbe in fruits, in vegetables [7], and in rice plant [8]. Moreover, *Stenotrophomonas maltophilia* has been reported as the virulent nosocomial strain affecting multiple organs especially the respiratory and urinary tract [9, 10]. However, the pharmacological and

toxicological manifestation of this microbe in brain and central nervous system is missing till date across any medical or scientific databases. To fill this gap, in this study, we have aimed to unravel the potential neuropharmacological alterations of *S. maltophilia* through both in vivo and bio-molecular landscape. To our knowledge, this is the first attempt to report the CNS toxicity of this rice contaminating microbe till date and a molecular insight to analyze the mechanism, pathway, and dynamics of this altered neuropharmacology.

Materials and Methods

Reagent and Chemicals

The chemicals dopamine hydrochloride (Dopaxin, Serum Institute, Conc: 200 mg/5 ml), noradrenaline (Noradria, Troikaa Parenteral Limited, Conc: 2 mg/ml), adrenaline (Adrenaline Injection, Reliance Formulation Pvt. Ltd., Conc: 1 mg/ml), serotonin (Sigma-Aldrich), γ-amino butyric acid-GABA (Sigma-Aldrich), and 3-mercaptopropionic acid (Sigma-Aldrich) were used for the study Dowex 50 W resin (X-4, 200–400 mesh) were procured from Sigma-Aldrich, while other chemicals such as perchloric acid, EDTA, K_2CO_3, NaOH, ethanol, HCL, and glass distilled water were procured from the analytical laboratory of Dr. B.C. Roy College of Pharmacy & Allied Health Sciences, Durgapur, WB, India. 10% v/v formaldehyde in normal saline, 70% alcohol, 90% alcohol, 100% alcohol, xylene, paraffin (Leica biosystems), eosin Y solution (Sigma-Aldrich), and hematoxylin solution (Sigma-Aldrich) were used for histopathological analyses.

Cooling centrifuge (Remi, Mumbai, Model: CM-8 Plus) was used for centrifugation throughout the process, tissue homogenizer (Prolab Scientific Instruments) was used to homogenize the brain tissue, and spectrofluorometer (Genesys Incorporation) was used to assess the fluorimetric measurement in this study.

Animal Collection, Acclimatization, and Ethical Approval

The adult Swiss albino mice of either sex weighing 18–22 g were taken for the studies which were maintained at the light/dark cycle at 22 ± 1 °C. For acclimatization, mice were housed a week prior to the study in the laboratory conditions. Food was withdrawn 2 h before dosing but water was available ad libitum.

Isolation and Identification of Bacterial Strain

The bacteria in contaminated rice was isolated and characterized by 16 s rRNA sequencing and Gram staining as *Stenotrophomonas maltophilia* which was reported earlier [6].

Preparation of Cell Lysate from S. maltophilia

In order to simulate boiled rice condition, the livestock of *S. maltophilia* culture containing a 10^5 CFU/ml of colony count was washed with 10 ml 0.9% NaCl, diluted up to 100 ml with same saline, and autoclaved at 121 °C for 15 min at 15 psi pressure. The autoclaved lysate was collected, cooled, and lyophilized to obtain powdered cell extract which was stored at 4 °C for further studies.

FAME Analysis

The chemical tests, related thin layer chromatography, SDS-PAGE, DNA gel electrophoresis, and other spectrophotometric investigations reported the absence of carbohydrate or protein molecules. FAME analysis was undertaken via GC–MS detection to estimate the fatty acid of *Stenotrophomonas maltophilia* (National Centre for Cell Science, Pune, India) as the cell membrane lipids or fatty acids were presumed to have such pharmacological effects. Briefly, the fatty acid of the bacteria, studied as the lipid in bacteria, is found as free fatty acids [11]. In this method, the whole cell fatty acid is hydrolyzed involving the fatty acids present in phospholipids, sterols, triacylglycerol, and other lipid molecules and was subsequently analyzed by the gas chromatography-mass spectrometry [12]. The esterification of carboxyl group with the methyl group was done and the methyl ester was used as the label for gas chromatography.

Dosage Preparation and Administration

The lyophilized bacterial cellular debris was dissolved in normal saline (0.9% w/v) and used for the dosing. The LD_{50} was determined in the previous study following the OECD guidelines 425 [6]. Subsequently, the animals were randomly grouped in five with 12 mice in each group and were distributed accordingly: group I—untreated control (received only the solvent), group II—received dose at 200 mg/kg *p.o.* for 15 days daily, group III—received dose at 300 mg/kg *p.o.* for 15 days daily, group IV—received dose at 200 mg/kg *p.o.* once a week for 3 months, and group V—received dose at 300 mg/kg *p.o.* once a week for 3 months.

Mechanism of Bioamine Level Alteration in Brain-Molecular Docking Analysis

In this study, we tried to unravel the molecular mechanism for bioamine level alteration in brain cell by the free fatty acid/s. We targeted three major bioamine synthetic pathways for the analysis, dopaminergic pathway, GABA synthetic pathway, and serotonin pathway. However, the dopaminergic and serotonergic pathways being similar with their nature of catalytic enzymes, the mechanistic dissection in this study was focussed on dopaminergic pathway only to avoid redundancy of approach. GABA synthetic pathway was undertaken as a fundamental pathway.

Molecular Docking Studies-Binding Mode Prediction of 18:1 ω 7 c Unsaturated Fatty Acid

For performing docking with the dopaminergic pathway enzymes, phenyl alanine 4-hydroxylase (PAH, PDB ID: 4AH), tyrosine 3-hydroxylase (TYH, PDB ID: 2XSN), DOPA decarboxylase (DDC, PDB ID: 1JS3), dopamine β-hydroxylase (DBH, PDB ID: 4ZEL), glutamate decarboxylase (GDC, PDB ID: 2OKJ), and GABA transaminase (GABA-T, PDB ID: 4Y0H) were downloaded from protein data bank (PDB). The inbound ligands and water molecules were removed from the proteins by Discovery Studio Visualizer (ver 4.2) (Accelrys, USA) and all the hydrogens were added on the protein. The processed molecule was further taken to AutoDock Tools (ver 4.2, Scripps Research Institute, La Jolla,

California, USA) and merged to the non-polar hydrogens. The Kollman and Gasteiger charges were added, the chargeless atoms were pointed out, and number of rotable bonds was assigned. The Torsional degree of freedom was also fixed for the protein. However, for ligand, the non-polar hydrogens were merged and all the bonds were allowed to rotate freely. The torsional angle and bond angles were also set to rotate freely. Finally, both protein and ligand structures were saved in PDBQT format compatible with AutoDockVina.

For docking, the protein molecule was confined to a grid box covering the whole molecule. The whole protein subunit was undertaken for docking in order to allow allosteric docking thus allowing all possible binding modes of the ligand. The protein was docked with two modes docking. First docking was performed with standard substrate as per the biosynthetic pathway or with co-crystallized ligand (if multiple binding sites were visible from PDB). The second docking was performed with the 18:1 ω 7 c unsaturated fatty acid in order to investigate any sort of binding site overlap that might interfere with the standard bio-molecule binding subsequently affecting the bioamine production.

Determination of IC_{50} and ROS of the Cell Lysate in Murine Brain Cell Lines

The cell lines used were C8-B4 (murine brain cells) which were obtained from the institutional facility (Chittaranjan National Cancer Institute, Kolkata, India). The cells were cultured in DMEM media (Dulbecco's Modified Eagle Media of pH 7.4) containing 10% FBS (fetal bovine serum, Gibco) supplemented with HEPES (Sigma) and penicillin–streptomycin (PEN-STREP) at 37 °C in an air jacketed CO_2 (5%) incubator and cells were allowed to get attached for 24 h before treatment.

In Vitro Toxicity Evaluation and Determination of IC_{50}

The effect of cell lysate on cellular cytotoxicity was measured in murine brain cell lines by MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) using EZcountTM MTT Cell Assay Kit from Himedia using manufacturer's protocol. Data was analyzed and evaluated in Excel software.

Reactive Oxygen Species Measurement

The reactive oxygen species (ROS) in normal murine cell lines was measured by DCFH-DA (dichloro-dihydro-fluorescein diacetate) staining method. 1×10^6 cells were incubated with DCFH-DA (10 μM) for 30 min at 37 °C in the dark. The fluorescence intensity of DCFH was measured using spectrofluorometer (Varian Cary).

Neurobehavioral Study *In Vivo*

Evaluation of Locomotor Activity-Actophotometer Assay

The locomotor activity was evaluated with actophotometer as per standard protocol [13]. The change in total number of moves was evaluated for cut-off time of 5 min.

Evaluation of CNS Depression-Forced Swim Test or Porsolt Swim Test

The forced swim test (FST) was carried out [14] with cut-off time of 6 min where the last 4 min were counted for the mobility/immobility time. The reduced mobility was the indication of depression as immobility is referred as the index for depression.

Determination of Brain Bioamine Levels

The bioamine levels in rat brain before and after administration of cell lysate were assessed as described below.

Column Preparation

The column was prepared in glass tube approximately of length of 5 cm and diameter of 4.0 mm after mixing the resin Dowex 50 W in phosphate buffer. The washing was done accordingly, at first, 20 ml of 2 N NaOH prepared in 1% EDTA was passed through the column, then washed with glass distilled water until neutral. After which 20 ml of 2 N HCI was passed through the column, then washed with glass distilled water until neutral.

Extraction of Dopamine, Serotonin, Adrenaline, and Noradrenaline [15]

The whole brain was collected in ice-cold condition. To about 2 g of whole brain, 10 ml of 0.4 N perchloric acid, 5% $Na_2S_2O_5$, and 0.2 ml 10% EDTA were added and then homogenized in ice-cold condition. The homogenate was centrifuged at 14,000 g for 10 min at 0 °C. The supernatant was collected and pH was adjusted to 2 with 5 N K_2CO_3.

For isolation and elution, the column was washed with glass distilled water and the sample was passed through it. Then washing was done with 15 ml of glass distilled water. The column was washed with 15 ml sodium phosphate buffer (pH = 6.5) followed by washing with 15 ml of glass distilled water. Through the column, 10.5 ml of N-aqueous-HCl was passed of which first 3.5 ml was discarded and the last 7 ml was used to study adrenaline and noradrenaline. Then the column was washed with 4.7 ml of N-ethanolic (50%)-HC1 where the last 3.5 ml was collected to study the dopamine and serotonin. The elution was done at a room temperature about 25–26 °C and in dark condition.

Extraction of GABA [16]

The mice were injected with 3-mercaptopropionic acid (100 mg/kg, ip) before scarification. To about 2 g of whole brain tissue collected in ice-cold condition, 10 ml of 0.4 M perchloric acid, 0.1 ml of 5% $Na_2S_20_5$, 0.2 ml 10% EDTA, and 0.1 ml 1 M citric acid were added. The tissue was homogenized in ice-cold condition and later centrifuged at 14,000 g for 10 min at 0 °C. The supernatant was collected and pH adjusted to 3 with 2.5 M K_2CO_3.

For isolation and elution, the sample was run through the column. Primarily, the column was washed with 8 ml of glass distilled water. Then, 8 ml of 0.025 M sodium citrate (pH 4.5) was passed through the column followed by equilibration with 4 ml of 0.05 M sodium

citrate buffer (pH 5.3–5.4). The first 0.5 ml was allowed to flow out and the later 3.5 ml was collected for the study.

Detection of GABA [16]

Ninhydrin reaction: The eluate was alkalinized with 50 µl of 2.5 M K_2CO_3. Then, 0.8 ml of the eluate was taken and 0.4 ml of ninhydrin solution was added. It was incubated at 60 °C for 30 min and then cooled for 30 min at room temperature. The fluorescence was read at 380 nm excitation and 450 nm emission.

Detection of Catecholamines [15]

For the detection of catecholamines, dopamine, serotonin the reagents that were added to the standard, control eluate and the sample eluate has been tabulated in the Table 1.

The fluorescence emission for different samples was read at the following wavelength for different catecholamines after UV irradiation for 5 min—dopamine was read at 330/375 nm, adrenaline was read at 330/410 nm, noradrenaline was read at 330/400 nm, and serotonin was read at 310/355 nm. All the data was normalized with respect to per gram of rat brain weight and final report is provided as the normalized fluorescence unit (FU) observed from detection of each bioamine.

Histopathology of Brain of Experimental Animals

The histopathology study of brain was conducted following the method of Carlton, 1967 [17, 18]. After treating male albino mice with 300 mg/kg *p.o.* for 3 months, the animals were sacrificed as per standard protocol of CPCSEA. The brain collected was washed with

Table 1 Concentration of reagents that were added for the fluorimetric determination of adrenaline, noradrenaline, dopamine, and serotonin (Atack, 1973)

Reagent	Standard (ml)	Reagent blank (ml)	Sample (ml)	Control (ml)
H_2O	–	0.05	0.05	0.05
4% EDTA	0.05	0.05	0.05	0.05
0.25% $K_3(Fe(CN)_6)$	–	–	–	0.05
3.225 NaOH in 0.125 M $Na_2S_2O_3$	–	–	–	0.4
Wait for more than 1 min				
Glacial acetic acid	–	–	–	0.2
Eluting solution	1.0	1.0	–	–
Eluate	–	–	1.0	1.0
Standard drug solution (1 µg/ml)	0.05	–	–	–
0.25% $K_3(Fe(CN)_6)$	0.05	0.05	–	–
Wait for 3–6 min				
3.225 NaOH in 0.125 M $Na_2S_2O_3$	0.4	0.4	0.4	–
Wait for 3–6 min				
Glacial acetic acid	0.2	0.2	0.2	

0.9% w/v normal saline and then excised. The liquid fixation was done in 10% v/v of formaldehyde solution in phosphate buffer. The routine techniques of dehydration, clearing, and wax infiltration were done followed by tissue embedding into paraffin. The tissue sections of 5 μm thickness were stained with eosin and hematoxylin. The photomicrograph was taken with Olympus Research Microscope (model BX51).

Statistical Analysis

Statistical analysis of the data was performed by both one- and two-way RM-ANOVA (Graph Pad Prism Version: 8.4.3) which is used to analyze matching across groups within different time range. In two-way ANOVA, analysis of variance was derived as a function of two independent variables or factors which reduced the variation of the residual factor to study the statistical significance of outputs. The parametric evaluations were taken as interactions between time range and groups; variability across different groups; variability among different time range; and residual variability or error existing in the model.

For ANOVA, p-value < 0.05 is considered as statistically significant. In our study, *** denotes $p < 0.001$, ** denotes $0.001 < p < 0.01$, and * designates $0.001 < p < 0.05$.

Results

Identification of the Microbial Strain Isolated from Contaminated Rice and LD_{50} of the Cell Debris and Lysate

The microbial strain isolated from contaminated rice was identified by 16 s rRNA technique as described earlier which was found to be *Stenotrophomonas maltophilia* and LD_{50} of the cell lysate was found to be more than 2000 mg/kg orally [6].

FAME Analysis

The FAME analysis was done in triplicates and the percentage of the available free fatty acid is given in Table 2 and the structure has been mentioned in Fig. 1 which are 18:1 ω 7 c (18.34%, Fig. 1a), 16:1 ω 6 c (5.49%, Fig. 1b), 16:1 ω 7 c (5.49%, Fig. 1c), and 18:2 ω 6,9 c (0.26%, Fig. 1d). The fatty acids are basically mono- or di-unsaturated long-chain fatty acids.

Table 2 The percentage of the available free fatty acid from the FAME analysis

SL. no	Free fatty acid	Percentage present
1	18:2 ω 6,9 c	0.26
2	16:1 ω 7 c	5.49
3	16:1 ω 6 c	5.49
4	18:1 ω 7 c	18.34

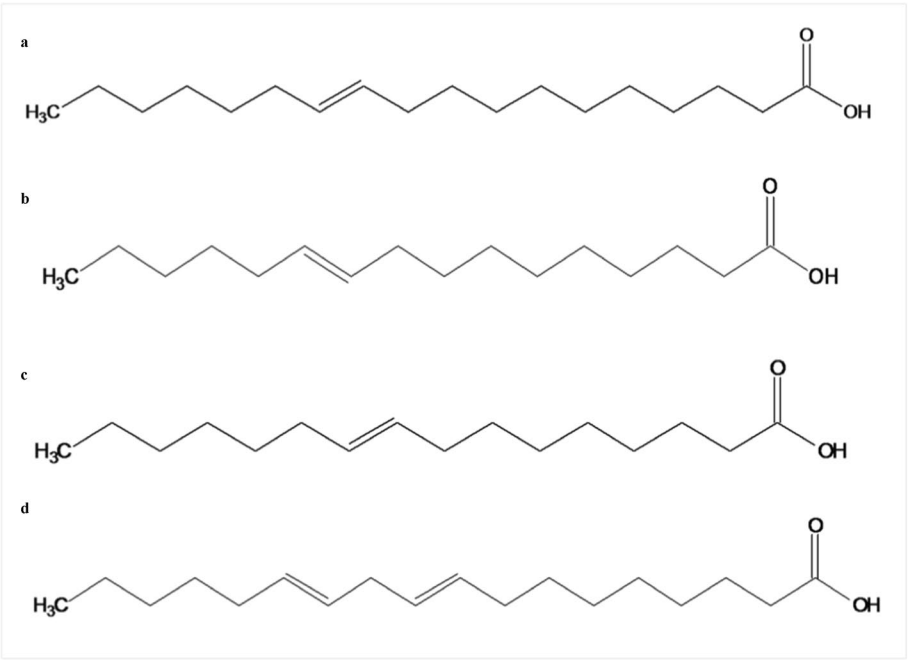


Fig. 1 Structure of free fatty acids obtained from cell lysate of *Stenotrophomonas maltophilia* **a** 18:1 ω 7 c, **b** 16:1 ω 6 c, **c** 16:1 ω 7 c, and **d** 18:2 ω 6,9 c

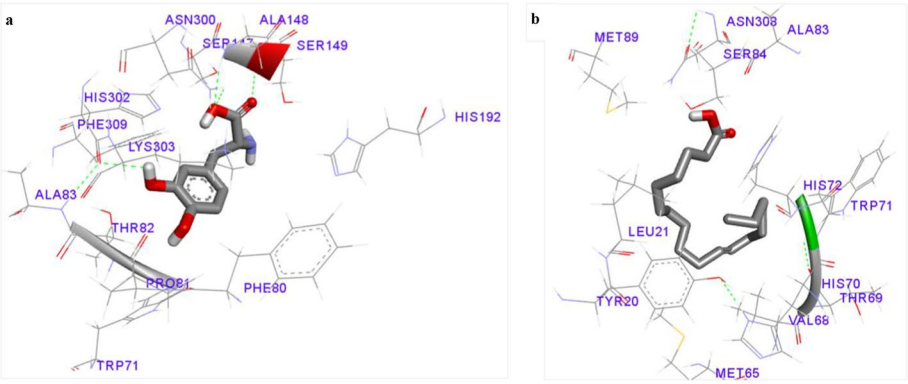


Fig. 2 Molecular docking analysis of **a** DOPA-DOPA decarboxylase and **b** 18:1 ω 7 c-DOPA decarboxylase. The binding site engulfed by ASN300, HIS302, PHE309, and LYS303 in DDC-DOPA complex (A) is intervened by the perturbation of 18:1 ω 7 c fatty acid at amino acid residue ASN308 (B). TRP71 in DDC-DOPA is pierced by the fatty acid which covered the binding site comprising of MET65, VAL68, THR69, HIS70, and TRP71. Furthermore, DDC-DOPA binding at PHE80, PRO81, THR82, and ALA83 is considerably intervened by the fatty acid binding at ALA83, SER84, and MET89 which are mutually inclusive with each other

Molecular Docking Studies

The docking of DDC-DOPA and DDC-18:1 ω 7 c fatty acid revealed a considerable overlapping of binding sites between DOPA and the fatty acid on DDC enzyme which was not seen in case of other bioaminergic enzymes such as PAH, TYH, or DBH (detailed data not shown). The DDC binding site was engulfed by ASN300, HIS302, PHE309, and LYS303 in DDC-DOPA complex (Fig. 2a) is intervened by the perturbation of 18:1 ω 7 c fatty acid at amino acid residue ASN308 (Fig. 2b). TRP71 in DDC-DOPA is pierced by the fatty acid which covered the binding site comprising of MET65, VAL68, THR69, HIS70, and TRP71. Furthermore, DDC-DOPA binding at PHE80, PRO81, THR82, and ALA83 is considerably intervened by the fatty acid binding at ALA83, SER84, and MET89 which are mutually inclusive with each other.

Analyzing GABA pathway revealed that 18:1 ω 7 c fatty acid interfered with GAD active site while compared with glutamic acid itself. For instance, the glutamic acid binding with HIS 291 (Fig. 3a) is shown to be intervened by fatty acid binding with a major binding groove posed by HIS291, TYR292, SER290, GLU288, and GLN289 at GAD (Fig. 3b). Further, THR348 and GLY346 could be of mutual interest of both glutamic acid and fatty acid binding where the latter established greater binding motif with THR347, THR348, VAL349, and TYR350 within GAD (Fig. 3b). In addition, glutamic acid binding with ARG567 (Fig. 3a) is suggested to be altered by 18:1 ω 7 c fatty acid binding with nearest binding motif composing of GLN557, PRO558, and GLN559 (Fig. 3b).

The IC_{50} and ROS Activity

The toxic manifestation of the *S. maltophilia* cell lysate was observed in the C8-B4 brain cell line (monocyte, macrophage microglia, spontaneously transformed cerebellum cell line) and percentage inhibition was measured over various doses at different time points.

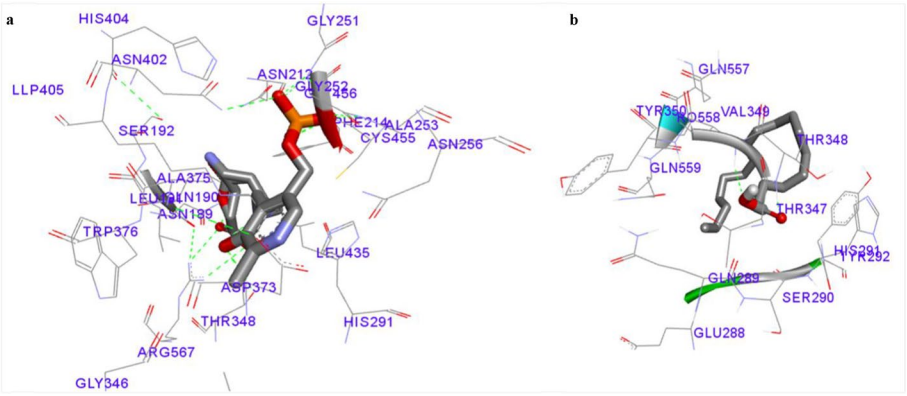


Fig. 3 Molecular docking analysis of **a** glutamic acid-glutamate decarboxylase, **b** 18:1 ω 7 c—glutamate decarboxylase. The glutamic acid binding with HIS 291 (7A) is shown to be intervened by fatty acid binding with a major binding groove posed by HIS291, TYR292, SER290, GLU288, and GLN289 at GAD (7B). Further, THR348 and GLY346 could be of mutual interest of both glutamic acid and fatty acid binding where the latter established greater binding motif with THR347, THR348, VAL349, and TYR350 within GAD

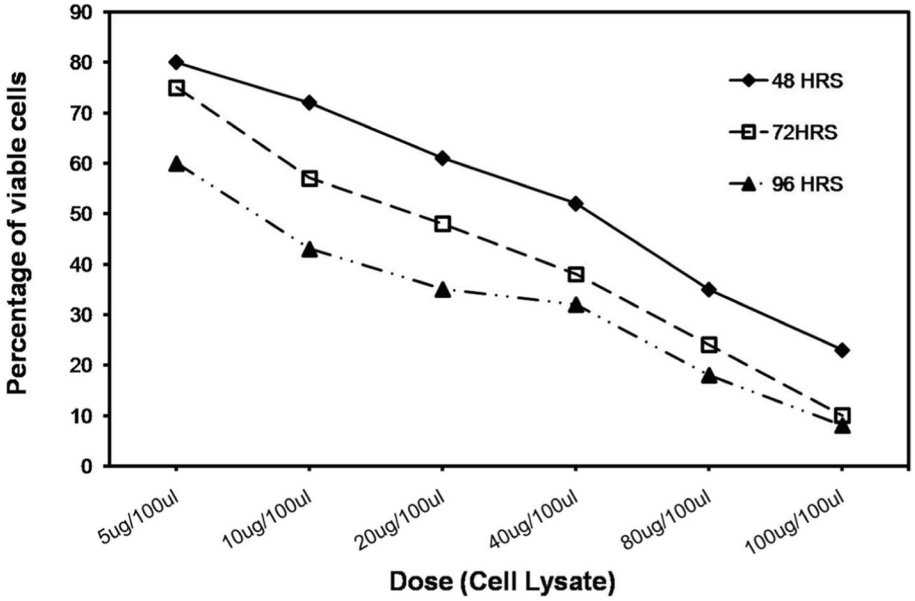


Fig. 4 Cell inhibition assay of *Stenotrophomonas maltophilia* lysate in C8-B4 cell line percentage of viable cells was counted exposing the murine C8-B4 cell line to *Stenotrophomonas maltophilia* cell lysate in dose- and time-dependent manner. When the dose was increased up to 100 µg/100 µl, the viable cells accounted only 23% at 48 h exposure. Furthermore, the time exposure being increased to 96 h with the same dose, the cell viability dropped drastically to 8%

At 5 µg/100 µl dose of the lysate, the viability of the cells was 80% at 48 h which dropped down to 60% on exposure up to 96 h (Fig. 4, IC_{50} 77 µg/100 µl for 48 h exposure). The % cell inhibition revealed a dose-dependent decrease by the bacterial cell lysate. Notably, when the dose was increased up to 100 µg/100 µl, the viable cells accounted only 23% at 48 h exposure. Furthermore, the time exposure being increased to 96 h, the cell viability dropped drastically to 8% (Fig. 4, IC_{50} 12 µg/100 µl, for 96 h exposure).

Concomitant with the finding of cell inhibition, a gradual increase in the ROS in the murine brain cells was found with time and concentration of bacterial cell lysate. Assuming untreated cell ROS level as baseline concentration, 5 µg/100 µl dose treatment increased the ROS level two times of baseline at 48 h exposure which amounted to 5.4 times on 96 h exposure (Fig. 5). Following the general pursuit of dose-dependent increase of response, when the dose was increased to 100 µg/100 µl, the ROS level increased to 5.6 times at 48 h exposure and 12.9 times at 96 h exposure.

The Changes Observed in Behavioral Profile

The behavioral profile changes in mice before and after administration of cell lysate as described below. The experimental animals were exposed to the cell debris and lysate of *Stenotrophomonas maltophilia* in two different studies with three different doses. The untreated control was administered with the blank solvent. This was done to develop the similar psychological condition as that of the lysate-treated group together with to assess

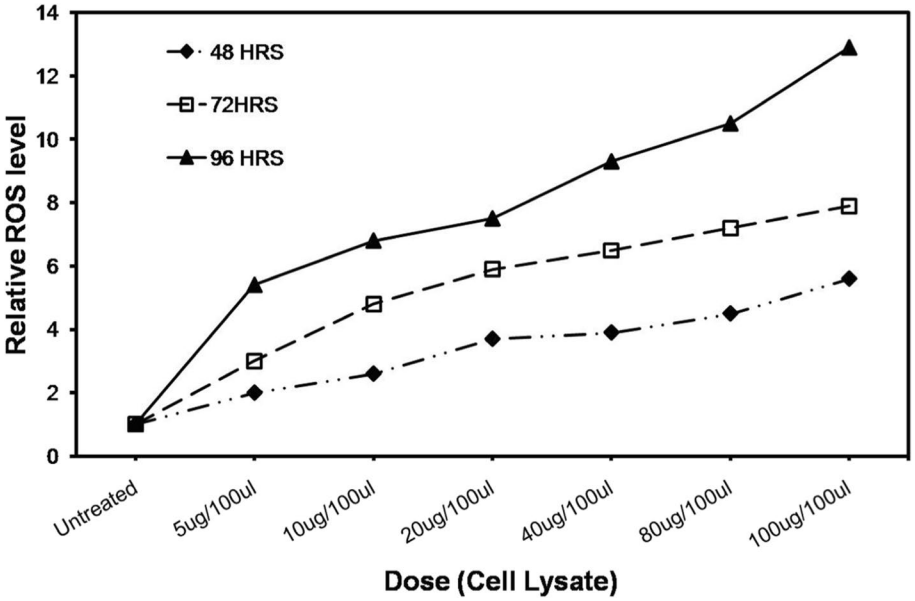


Fig. 5 The relative ROS level on murine brain cells in C8-B4 after exposure to *Stenotrophomonas maltophilia* lysate in C8-B4 cell line. The ROS level was measured in cell line in dose- and time-dependent manner of cell lysate. Following the dose-dependent increase of response, when the dose was increased to 100 µg/100 µl, the ROS level increased to 5.6 times at 48 h exposure and 12.9 times at 96 h exposure

any baseline pharmacological effect caused due to the solvent apart from the lysate under investigation. As in the study, the neurobehavioral pharmacology was evaluated.

Estimation of Locomotor Activity

It has been observed that the acute exposure at low dose (200 mg/kg *p.o.*) did not show any significant change in the locomotor activity initially (Table 3) but the reduction in the locomotor activity was observed from day 7 (67.44%, $p < 0.05$) which showed high significance (55.52%, $p < 0.001$) from day 8 to 15 of treatment. On the contrary, the high dose exposure (300 mg/kg *p.o.*) reduced the locomotor activity with extreme significance (58.51%, $p < 0.001$) from day 1 itself and reduced prominently till day 15 compared to the untreated animals (39.72%, Table 3). Besides, in the 3 months study as mentioned in Table 3, a reduction in the locomotor activity was observed from 8th week for low dose (200 mg/kg) which became highly significant (57.79%, $p < 0.001$) from 11th week exposure as compared to the untreated animals. Increasing dose from low to high (300 mg/kg), the reduction of locomotor activity was observed from 1st week of study which further got reduced on continuous exposure for 3 months as given in Table 3 (38.50%, $p < 0.001$).

Estimation of Depression

The depression was observed for both acute and chronic treatment of the cell debris and lysate of *Stenotrophomonas maltophilia* that has been presented in Table 4. The immobility of experimental animals as considered as to be the index for depression

Table 3 Estimation of locomotor activity of *Stenotrophomonas maltophilia* cell debris and lysate

Day	Group I (control untreated)	Group II (dose 200 mg/kg *p.o.* 15 days)	Group III (dose 300 mg/kg *p.o.* 15 days)
Administration for 15 days			
1	331.5 ± 7.17	212.66 ± 8.77	199.54 ± 6.63**
2	335.5 ± 5.66	214.16 ± 9.28	200.56 ± 7.72**
3	316.5 ± 5.09	227.33 ± 5.80	189.33 ± 5.80**
4	313.83 ± 8.33	243 ± 6.07	178 ± 7.06**
5	296 ± 6.23	216.6 ± 4.58	187.66 ± 3.85*
6	287 ± 5.83	197.56 ± 6.54	171.22 ± 6.63**
7	285.83 ± 6.33	222.5 ± 9.24*	165.48 ± 7.73*
8	301.66 ± 8.29	203.44 ± 3.39**	167.5 ± 3.08**
9	318.33 ± 6.89	202.83 ± 4.45**	152.66 ± 3.96**
10	303.5 ± 3.78	203.16 ± 5.98**	148.96 ± 5.52**
11	311.16 ± 9.81	194.33 ± 6.08**	140.24 ± 8.38**
12	305.66 ± 8.33	195.66 ± 8.31**	145.22 ± 7.85**
13	298.66 ± 5.56	183.64 ± 6.24**	137.48 ± 5.56**
14	315.28 ± 9.66	177.56 ± 8.55**	128.22 ± 3.49**
15	298.76 ± 5.56	174.82 ± 6.74**	118.66 ± 3.78**

Week	Group I (control untreated)	Group IV (dose 200 mg/kg *p.o.* 3 months)	Group V (dose 300 mg/kg *p.o.* 3 months)
Administration for 3 months			
1	300.5 ± 5.64	256.66 ± 7.75	185.66 ± 8.23**
2	298.56 ± 6.65	250.22 ± 3.32	182.34 ± 8.83**
3	323.24 ± 3.32	236.64 ± 7.85	187.68 ± 6.63**
4	298.54 ± 6.65	240.56 ± 9.92	165.22 ± 5.52**
5	300.54 ± 3.24	235.62 ± 3.36	156.84 ± 7.79**
6	292.56 ± 5.78	221.22 ± 4.45	150.42 ± 54.52**
7	290.66 ± 4.45	210.36 ± 5.52	136.24 ± 5.78**

Table 3 (continued)

Day	Group I (control untreated)	Group II (dose 200 mg/kg *p.o.* 15 days)	Group III (dose 300 mg/kg *p.o.* 15 days)
8	283.12 ± 5.89	195.42 ± 8.23*	122.56 ± 3.65**
9	280 ± 5.23	188.52 ± 4.12*	115.48 ± 8.45**
10	271.44 ± 6.65	175.78 ± 6.63*	108.06 ± 6.22**
11	275.66 ± 4.32	169.24 ± 4.41**	112.54 ± 7.72**
12	262.08 ± 5.53	151.48 ± 8.85**	100.88 ± 5.80**

All values are mean ± SD, $n = 5$, *$p < 0.05$, **$p < 0.001$ when compared with control

Same animals were used as control and drug treated

Table 4 Estimation of depression (immobile time) of *Stenotrophomonas maltophilia* cell debris and lysate

Day	Immobility time in sec		
	Group I (control untreated)	Group II (dose 200 mg/kg *p.o.* 15 days)	Group III (dose 300 mg/kg *p.o.* 15 days)
Administration for 15 days			
1	43.66 ± 3.53	65.66 ± 2.31	91.56 ± 4.67**
2	57 ± 7.41	71.55 ± 6.61*	90.66 ± 5.64**
3	41.16 ± 5.79	70.80 ± 9.25**	92.5 ± 5.31*
4	48.33 ± 7.09	73.26 ± 5.59**	97.5 ± 8.16**
5	52.66 ± 5.23	72.46 ± 3.01*	94.83 ± 7.83**
6	44.16 ± 3.84	76.78 ± 4.31**	96.83 ± 2.84**
7	47.18 ± 5.04	80.36 ± 2.53**	96.83 ± 5.15**
8	49.58 ± 2.89	83.44 ± 6.23**	97.83 ± 4.67**
9	52.28 ± 6.63	84.84 ± 7.56**	100.5 ± 4.36**
10	49.68 ± 7.74	82.16 ± 3.71**	98.5 ± 6.19**
11	45.26 ± 6.95	81.5 ± 4.88*	100.83 ± 5.58**
12	50.28 ± 4.03	86.83 ± 4.70**	107.16 ± 3.47**
13	48.26 ± 5.08	89.5 ± 2.66**	109.66 ± 5.43**
14	54.28 ± 6.65	95.66 ± 4.05**	109.24 ± 5.33**
15	50.23 ± 8.87	100.53 ± 6.63**	115.40 ± 8.52**
Immobility time in sec			
Week	Group I (control untreated)	Group IV (dose 200 mg/kg *p.o.* 3 months)	Group V (dose 300 mg/kg *p.o.* 3 months)
Administration for 3 months			
1	43.66 ± 3.53	70.5 ± 3.44*	81.00 ± 1.85**
2	51.33 ± 2.36	78.83 ± 3.86**	83.40 ± 10.20**
3	57 ± 7.41	82.83 ± 4.07**	98.80 ± 1.2**
4	62 ± 6.63	89.5 ± 3.98**	133.24 ± 1.49**
5	41.16 ± 5.79	72.5 ± 3.93*	135.26 ± 6.36**
6	42.83 ± 5.95	70.83 ± 3.37*	144.26 ± 5.78**

Table 4 (continued)

Day	Immobility time in sec		
	Group I (control untreated)	Group II (dose 200 mg/kg *p.o.* 15 days)	Group III (dose 300 mg/kg *p.o.* 15 days)
7	46.83 ± 4.83	73.16 ± 4.40*	145.82 ± 2.41**
8	48.33 ± 7.09	87 ± 5.32**	139.65 ± 5.53**
9	52.66 ± 5.23	89.56 ± 6.33**	148.84 ± 4.23**
10	39.5 ± 4.43	96.34 ± 7.51**	151.26 ± 2.47**
11	44.16 ± 3.84	102.36 ± 6.03**	187.28 ± 3.62**
12	45.33 ± 12	100.68 ± 7.51**	195.62 ± 7.84**

All values are mean ± SD, $n = 5$, *$p < 0.05$, **$p < 0.001$when compared with control

Same animals were used as control and drug treated

accordingly it was found that with increased dose and time, there was gradual increase in the depression in experimental animals. Table 4 represents the result of acute study which showed that at low dose, depression developed significantly ($p < 0.05$) from day 2 (immobilization time increased 150.39%) onwards which became highly significant ($p < 0.001$) as compared to the untreated animals from day 8 (immobilization time increased 200.14%) but the higher dose developed depression from day 1 (immobilization time increased 209.71%) of exposure which was highly significant ($p < 0.001$) as compared to the untreated control. Whereas in the 3 months of study, it was observed that the depression at low dose was already significant in week 1 ($p < 0.05$) and became highly significant (immobilization time increased 222.15% $p < 0.001$) from 8th week of treatment as compared to untreated control (Table 4). However, a reduction in the locomotor activity was observed ($p < 0.001$) from 1st week of high dose treatment that increased along with the administration for 3 months as compared to the untreated animals (immobilization time increased 431.55%).

The Brain Bioamine Levels

In the present work, the cell lysate and debris from *Stenotrophomonas maltophilia* altered certain behavioral activities (locomotor activity and depression) in the experimental animals which are controlled by the central nervous system where bioamines play a major role. Thus, the bioamine levels were observed for acute and chronic study of two different treatment groups (200 mg/kg *p.o.* and 300 mg/kg *p.o.*) which is given in Figs. 6, 7, and 8. The dopamine level given in Fig. 6a showed that at low dose (200 mg/kg *p.o.*), there was a significant (71.43%, $p < 0.05$) decrease as compared to the untreated animals whereas the decrease of dopamine levels for acute high dose (62.85%, 300 mg/kg *p.o.*) was more intense. Similarly, in both the doses for chronic study, the brain dopamine depletion was highly significant (60.00–45.71% for low and high dose, respectively, $p < 0.001$). In Figs. 6b, 7a, and 7b, the brain noradrenaline, adrenaline, and serotonin levels have been summarized respectively. Both noradrenaline and adrenaline declined significantly (71.43–62.50% for low and high dose, respectively, $p < 0.05$) in treated animals compared to control; the difference becoming highly significant ($p < 0.001$) on increasing the doses for both acute and chronic treatment. Serotonin, which is another important bioamine, did not change significantly in the acute study whereas an extremely significant ($p < 0.001$) change was observed between treated and untreated groups in the chronic study (74.35% at 200 mg/kg and 53.81% at 300 mg/kg). Figure 8 shows that GABA, although did not reduce significantly at the acute condition, reduced with marked significance ($p < 0.001$) compared to control on chronic treatment with *Stenotrophomonas maltophilia* cell lysate (55.17% at 200 mg/kg and 46.55% at 300 mg/kg).

Alterations in the Brain Tissue of Experimental Animals

The histology of brain tissue after chronic administration of the cell lysate at higher concentration showed polymorphonuclear leucocytes in remarkable concentrations (Fig. 9b) (marked with red arrow) as compared to the brain tissue of experimental animals without cell lysate administration (Fig. 9a).

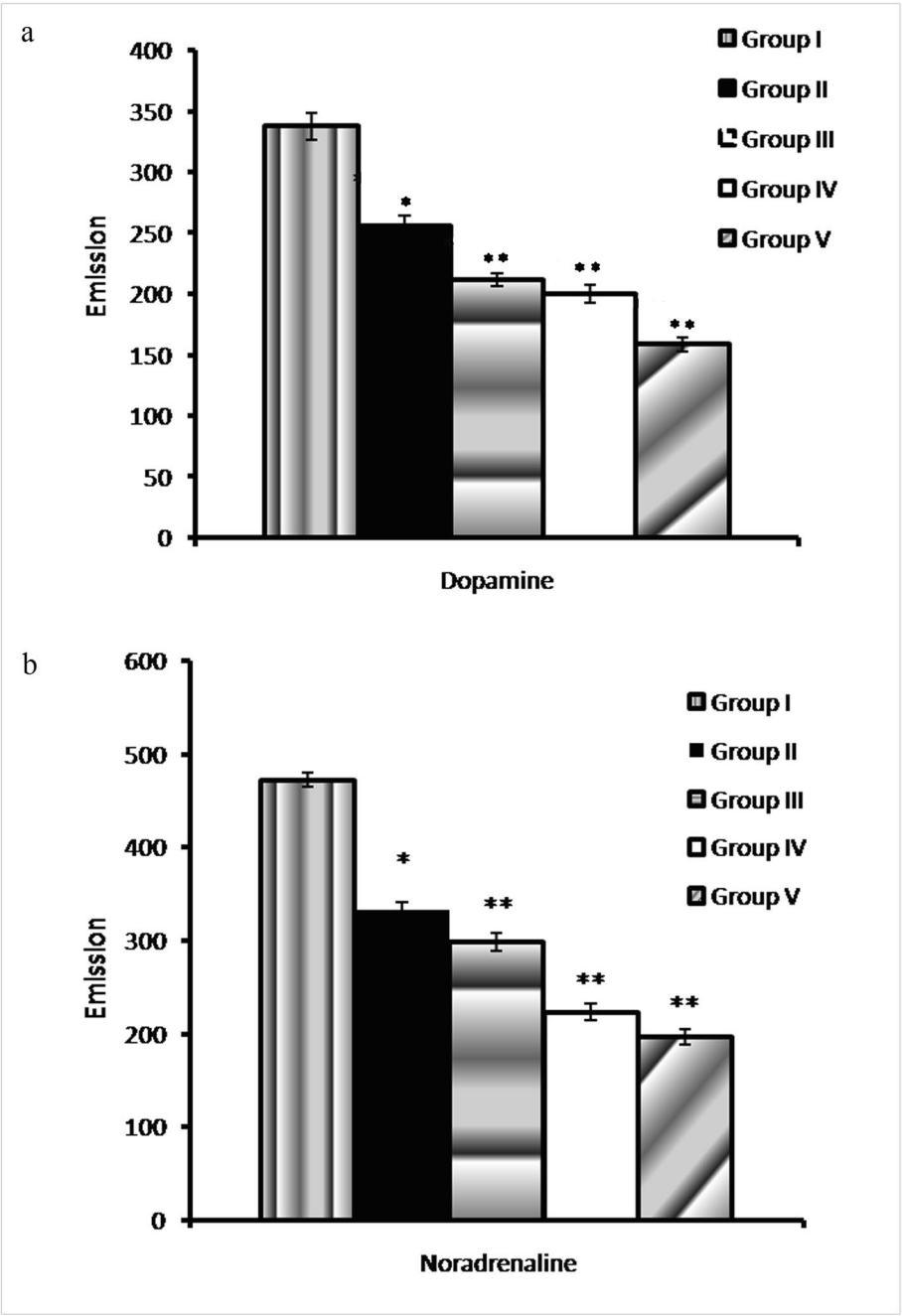
Fig. 6 The changes in the bioamine levels after administration of cell debris and lysate of *Stenotrophomonas maltophilia* at various doses and for different period: **a** dopamine level and **b** noradrenaline level. Both the bioamine levels were found reduced significantly in brain after treatment with cell lysate and the reductions got aggravated with increase of time and dose of treatment. The dopamine level given showed that at low dose (200 mg/kg *p.o.*), there was a significant (71.43%, $p < 0.05$) decrease as compared to the untreated animals whereas the decrease of dopamine levels for high dose (62.85%, 300 mg/kg *p.o.*) in acute study and in both the doses for chronic study was highly significant (60.00–45.71% for low and high dose, respectively, $p < 0.001$). The reduction in noradrenaline level was found up to 62.50% at 300 mg/kg dose in acute study

Discussion

Microbial contaminations from food are usually known to cause gastrointestinal disorders and visceral organ toxicity; however, alterations of neuropharmacological profiles from such contamination are rarely reported. The first novel finding of our study is the food microbial contamination altering neuropharmacological profile. Most molecules after ingestion are absorbed in the blood circulation from gastrointestinal tract but rarely reaches the brain or related cells crossing blood brain barrier. Interestingly in this work, the effect of the cell debris and lysate from *Stenotrophomonas maltophilia* showed a clear alteration in the behavioral activities in the animals and also a decrease in the brain bioamine levels. Therefore, it may be predicted that the cell debris of *Stenotrophomonas maltophilia* influenced the brain indicating that it can cross the blood brain barrier affecting brain-gut-enteric microbiota axis [19].

Since the boiled cell lysate of *Stenotrophomonas maltophilia* caused the pharmacological alterations, it can be assumed that carbohydrate or proteins may be dissociated or denatured at this condition eliminating the possibilities of such antigenic interference for such infection. Hence it can be hypothesized that the very microbe being a Gram-negative bacillus, highly thermostable, and resistant endotoxins may be the causative agent for such manifestation [11]. Lipid A is the component of endotoxins of Gram-negative organisms and is structurally present in the fatty acid portion [20] of bacterial cell membranes. Hence the question arose, has such endotoxins been present in the boiled cell lysate of *Stenotrophomonas maltophilia*? The FAME analysis revealed that the lysate contained long-chain unsaturated fatty acids which are possibly the degradation products of cell membrane lipids. The thermostable nature of endotoxins needs long treatment at high temperature to get destroyed [21]. Therefore, the cellular lysate and debris were obtained through wet heating (autoclave condition was maintained at 121 °C for 15 min at 15 psi pressure) for few minutes over 100 °C that is used in household cooking conditions which may retain the endotoxins and may be responsible for altered activities.

In the initial study, Chattopadhyay et al. [6] showed reduction in the locomotor activity so docking studies were done that revealed 18:1 ω 7 c fatty acid derived from bacterial cell lysate interferes with the binding site of DOPA decarboxylase suggesting that conversion of DOPA to dopamine gets reduced in the adrenergic pathway. We also presume that this interference subsequently deters in other bioamines production such as adrenaline and noradrenaline. The in silico analysis of GABA pathway revealed that free fatty acid might occupy the binding site of glutamate decarboxylase which is responsible for conversion of glutamic acid to GABA thus lowering the production of GABA. Thus, the unsaturated fatty acids most probably deter the decarboxylation reactions of various bioamine synthetic pathways. Since serotonin synthetic pathway is analogous to dopaminergic pathway, so there may be depletion of serotonin like a similar decarboxylation reaction suggested for other systems.



As the lysate crossed the blood brain barrier as there was reduced locomotor activity so the study was done on C8-B4 cell lines which revealed increment of the ROS level that may have caused oxidative stress. The cell inhibition assay on brain murine cells also revealed dose-dependent decrease of cell viability suggesting toxic effect of the lysate on

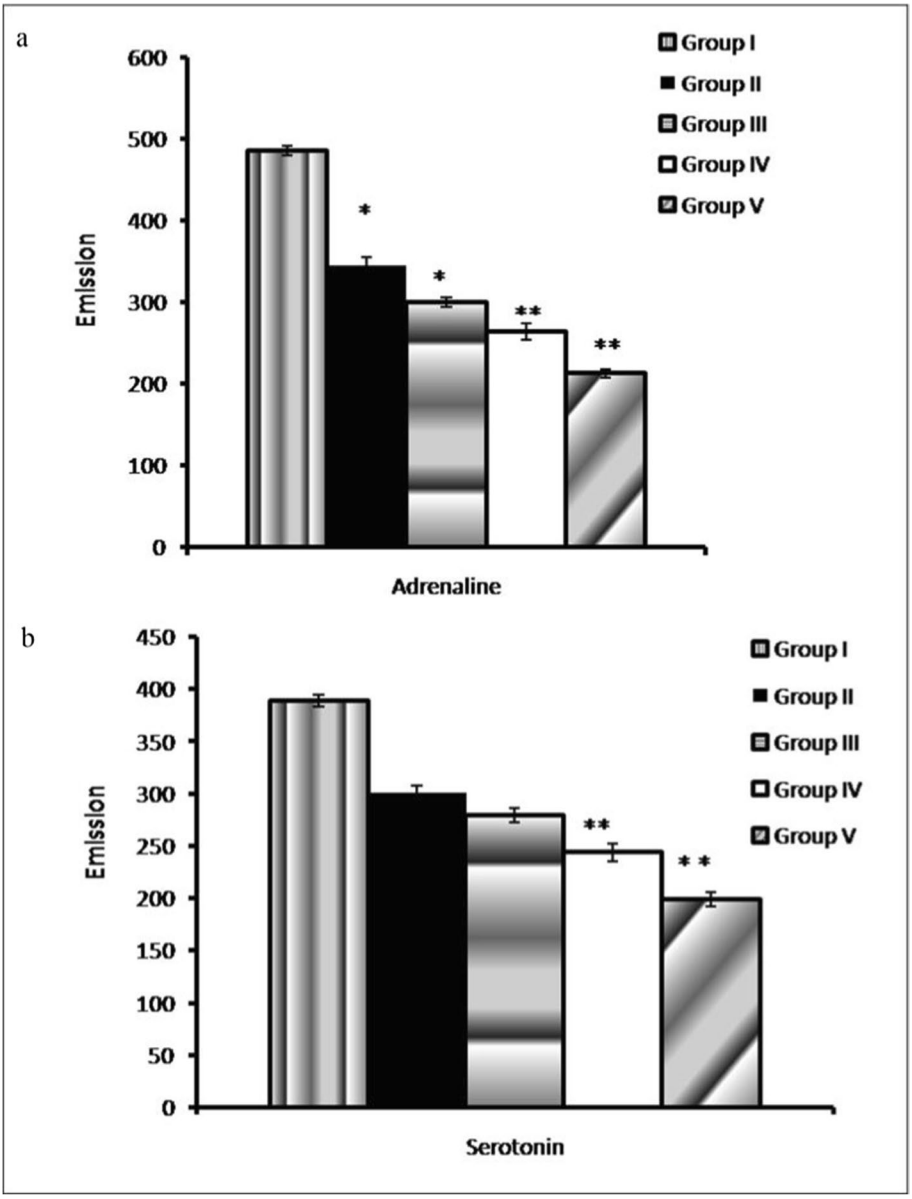


Fig. 7 Brain bioamine changes after administration of *Stenotrophomonas maltophilia* lysate at various doses and for different period: **a** adrenaline and **b** serotonin. Adrenaline declined significantly (71.43–62.50% for low and high dose, respectively, $p < 0.05$) in treated animals compared to control; the difference becoming highly significant ($p < 0.001$) on increasing the doses for both acute and chronic treatment. Serotonin, which is another important bioamine, did not change significantly in the acute study whereas an extremely significant ($p < 0.001$) change was observed between treated and untreated groups in the chronic study (74.35% at 200 mg/kg and 53.81% at 300 mg/kg)

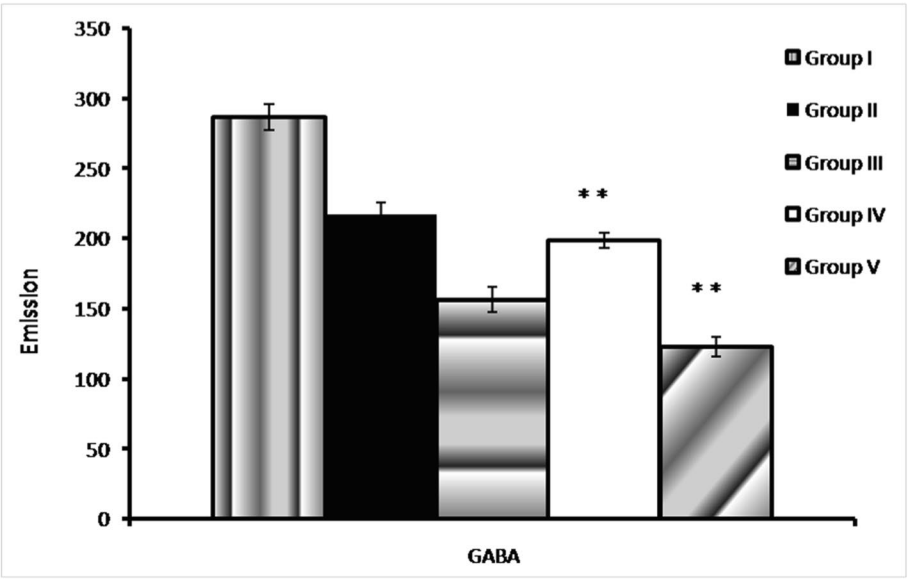


Fig. 8 Gamma amino butyric acid (GABA) profile in rat brain after *Stenotrophomonas maltophilia* lysate administration GABA, although did not reduce significantly at the acute condition, reduced with marked significance ($p < 0.001$) compared to control on chronic treatment with *Stenotrophomonas maltophilia* cell lysate (55.17% at 200 mg/kg and 46.55% at 300 mg/kg)

the microglial cells which promptly changes the microenvironment and among various factors, alterations in glutamate, ROS and RNS generation, or depletion of neurotransmitter supply has occurred as elicited by bioamine profiling [22]. Moreover, microglial cell lines (such as C8-B4) are responsible for neuroprotection, integrity of synaptic junction, maintaining neuronal excitation, neuronal debris removal, anti-apoptic activity during brain development, and brain protection and repair. Thus, microglial cytotoxic may have indulged in damaging output over brain, neuronal junction, or synaptic transmission leading to potential neurobehavioral alterations on subjects.

The locomotor activity is a function of neurotransmitters of CNS where the coordination is controlled by the dopaminergic system of CNS. With the reduction in the locomotor activity, the extract may have affected the coordination between cortical and subcortical dopaminergic structures of the brain which include dopamine of corpus striatum, globus-pallidus, subthalamic nucleus, and substantia nigra of the basal ganglia [23].

There are multiple causes that are responsible for reduction in the locomotor activity. Depression is one of the causes of reduced locomotor activity so in the present work, the principle neurobehavioral changes observed is depression [24]. The development of depression may be a cause of reduced locomotor activity. So the brain bioamine levels were determined after 15 days and 3 months of cell debris and lysate exposure as brain bioamines control the neurobehavioral activities [25, 26]. The results revealed a decreased level of all the bioamines-dopamine, noradrenaline, adrenaline, serotonin, and GABA suggesting that the cell lysate might have an inhibitory action on the brain bioamine basal profile. Increased depression may also be the result of GABA depletion; as there are reports of $GABA_B$ dysfunction in depression. Moreover, GABA produces a calming effect between the neurons due to $GABA_A$ receptor (Cl^- gated ion channel)-GABA interaction relieving

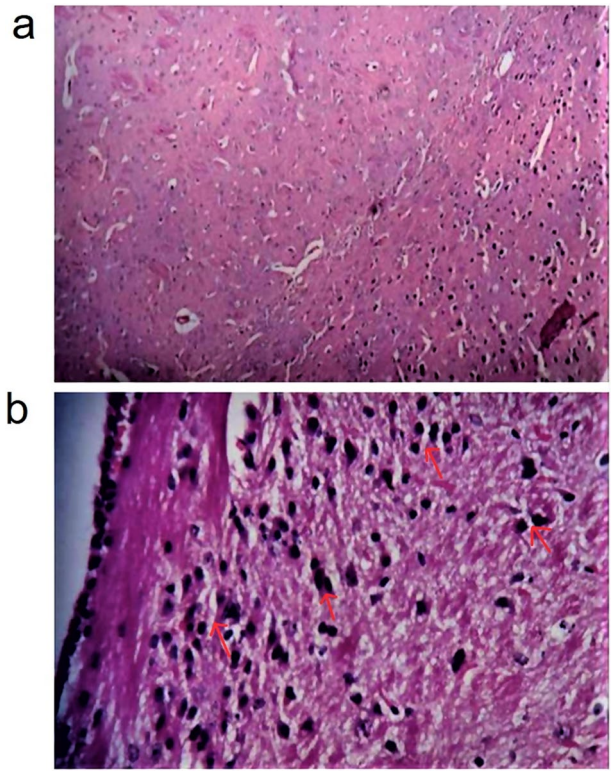


Fig. 9 Histopathological analysis of brain **a** without treatment to *Stenotrophomonas maltophilia* cell lysate and **b** treated with 300 mg/kg *p.o.* cell lysate for 3 months. The images were captured at 10 × magnification. The chronic exposure of *Stenotrophomonas maltophilia* cell lysate may have increased polymorphonuclear leucocytes in rat brain as revealed in **b**

depression [27, 28]. Thus, it is pertinent to believe that GABA depletion has resulted in increase of depression like behavior in the test animals. The increased depression, simultaneously, might lead to reduced locomotor activity [29]. Usually, it has been observed that dopaminergic pathway is under the inhibitory control of GABAergic neurons [30]. But here it has been observed that the lysate was capable to damage all bioamine pathways so the activity of the pathways is altered.

The brain dopamine, noradrenaline, and adrenaline levels progressively reduced on continuous exposure may be due to accumulation of fluid in the brain generating oxidative stress through free radical formation thereby affecting the neurobehavioral parameters [31]. The dopamine loss may be of cerebral perfusion pressure as of which the dopamine-secreting cells may got destroyed in the basal ganglia and also the loss of synapse at the cortex, brainstem, and basal ganglia causing neuropsychiatric symptoms [32]. Dopamine is the precursor of adrenaline and noradrenaline; therefore, low levels of dopamine by cell lysate and debris of *Stenotrophomonas maltophilia* may have resulted in lowering other brain amines [33].

In this present work, the chronic exposure to the cell lysate of *Stenotrophomonas maltophilia* may have caused ischemic condition in the brain as of which polymorphonuclear leucocytes infiltration increased [34]. The other causes for increased polymorphonuclear leucocytes may be trauma or infection [35] due to increased ROS level in the brain.

Thus, the present study reflected that the cell lysate of *Stenotrophomonas maltophilia* was responsible for CNS-enzymatic inhibition as revealed by docking studies,

microenvironment changes according to in vitro studies, changes in behavior, the brain bioamine levels, and in the alterations of the brain tissue histology. The very microbe has been obtained from contaminated rice, thus consumption of such rice even after boiling may lead to such molecular changes in CNS, brain tissues, and associated cells which ultimately may lead to potentially dangerous pathophysiological condition of the subject. Although further studies may be required, use of antidepressants or injection of dopamine or adrenaline at regular intervals may help the subject relieve such conditions.

Conclusion

The present work, with the cell lysate and debris of *Stenotrophomonas maltophilia*, a contaminant of rice, is the first attempt to evaluate about the hazardous effect of a contaminant on body system remains even after cooking. The lysate effectively changed the neurobehavioral activities by reducing locomotor activity due to increased depression with alteration in brain bioamine levels among the experimental animals. This indicated that the cell lysate and debris of the Gram-negative bacillus can easily cross the blood brain barrier and may lead to toxic effects in the central nervous system by developing neuropsychiatric problems if consumed continuously. The dose-dependent inhibition of murine brain microglial cells, concomitant rise in intracellular ROS level, indicated the possible change in microenvironment by the cell lysate and debris. The potential bioamine depletions in brain and docking guided enzymatic inhibitions of compounds characterized from bacterial cell lysate. In addition, the abnormal increase in the polymorphonuclear leucocytes in the brain tissue also reflected that chronic exposure to such contaminant may develop inflammatory condition in the brain affecting the body activity. This work may be further elaborated to study about the effect on the specified cell lines responsible for neurotransmitter release and treatment for the diseases caused by the cell remnants of *Stenotrophomonas maltophilia*. Although several nosocomial infections of *Stenotrophomonas maltophilia* have been reported earlier, to our knowledge, this is the first attempt to evaluate its neurotoxic behavior in quantitative level together with its contribution in contaminated rice generated brain toxicity prevalent in many parts of the world. Therefore, it may be concluded that the cell lysate of *Stenotrophomonas maltophilia* which efficiently crosses the blood brain barrier causing depression with simultaneous reduction in locomotor activity may be corrected by stabilizing the levels of brain bioamines as they were found to get reduced.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12010-022-03810-1.

Acknowledgements We are thankful to the NCCS (National Centre for Cell Science), Pune, India, for helping us in finding the free fatty acid present in bacteria and Bose Institute, Kolkata, India, to carry out the analytical work in their Central Instrumentation Facility. We are also thankful to the CPCSEA approved animal house of Dr. B.C. Roy College of Pharmacy & Allied Health Sciences, Durgapur, WB, India, for providing us the facility for conducting the animal studies in the research.

Author Contribution Experimental investigations (animal studies, bioamine determination)—Moitreyee Chattopadhyay, Dibyajyoti Das; work plan and conceptualization, molecular docking analyses, bioamine determination—Souvik Basak; data interpretation, original writing—Moitreyee Chattopadhyay, Souvik Basak; cell culture assays, data interpretation—Atish Barua; planning and guiding—Malaya Gupta, Gautam Kumar Bagchi; statistical analyses—Tanushree Karmakar.

Availability of Data and Materials All data and materials support published claims and comply with field standards.

Declarations

Ethics Approval The experimentation on laboratory animals was conducted according to the regulations of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) in India and the protocol was approved by Institutional Animal Ethics Committee of Dr. B.C. Roy College of Pharmacy and Allied Sciences, Durgapur, India (Approval Number: BCRCP/IAEC/1/2014).

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Competing Interests The authors declare no competing interests.

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