An in vitro approach to combat multidrug resistance in *Salmonella typhi* and human colon cancer with *Excoecaria agallocha* L. extract

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**Abstract**

**Background:** The incidence of antibacterial resistance and colon cancer is increasing in India. Antibacterial resistance and chemoresistance demand the need of developing herbal or natural chemotherapeutic agents. Our study thus, aims to determine the antibacterial and anticancer activities of the leaf extracts of the mangrove *Excoecaria agallocha*.

**Results:** Liquid chromatography–mass spectroscopy analysis of the ethanolic *E. agallocha* extracts revealed the presence of Bergenin. The plant extract fraction containing Bergenin had potent antibacterial action against a resistant strain of *Salmonella typhi* with an MIC value of 15.7 ± 0.04 µg/mL. Treatment of the bacteria with the plant extract made it moderately susceptible to the antibacterial drugs ampicillin, aztreonam, cefotaxime, chloramphenicol and imipenem. The plant extract caused membrane damage and disrupted the expression of a 33 kDa outer membrane protein (OmpA) in *S. typhi*. It was plausibly due to this mechanism of the plant extract that made the bacteria susceptible to the antibacterial drugs to a certain extent. Further, fluorescence microscopy analysis revealed the anticancer property of the extract against a human colon cancer (DLD-1) cell line by activation of Caspase-3 followed by subsequent apoptosis and exhibited cytotoxicity against the cancerous cell line with an IC50 value of 17.99 ± 1.12 µg/mL. Caspase-3 activity was observed to increase in a dose-dependent manner as determined by spectrophotometric assays. Moreover, the expression of the metalloproteinase-7 (MMP-7) was significantly reduced in plant extract treated DLD-1 colon cancer cells.

**Conclusion:** The results indicate that *E. agallocha* is a novel source of Bergenin, and the plant extract fraction under study may be used in combination therapy along with antibacterial drugs to combat antibacterial resistance of *S. typhi* and also to alleviate the risks of colon cancers in human. However, further investigations may be undertaken for its therapeutic application and to explore its potential bioactivity against other bacterial strains and human cancer cell lines.

**Keywords:** Antibacterial resistance, Bergenin, Caspase-3, Colon cancer, *Excoecaria agallocha*, Mangrove, *Salmonella typhi*
and Mycobacterium tuberculosis have exhibited resistance against antibacterials all over the world. In India, water and food borne infections are a common source of bacterial infection among individuals of all ages. Salmonella is one of the key contributors of such infections (Britto et al. 2019). While Salmonella typhi causes typhoid fever, other species such as S. enteritidis and S. typhimurium cause gastroenteritis and food poisoning. Recently, Salmonella typhi strains have shown resistance against antibacterial drugs such as fluoroquinolones, chloramphenicol, ampicillin, azithromycin and co-trimoxazole, as declared in the Geneva convention 2017 (Summary of the October 2017), that are clinically used to treat Salmonella infections (Khan and Musharraf 2004). This has led to complications in treatment procedures and increased mortality rate among infected patients. Further, records and literature study indicate the challenges due to emerging resistant strains and poor response to traditional, rendering the treatment strategy inefficient (Mylona et al. 2021). To aggravate the problem, severe Salmonella infections often are leading to gastrointestinal and colon cancer (Dujister et al. 2021).

Colon cancer is the third most common cancer among men globally and lately, an upsurge in the cases has been observed all over Asia including India (Ferlay et al. 2010). Though the reported cases in India are low, but an increase in colon cancer is anticipated due to the culminated factors of sedentary lifestyle and change in dietary patterns accompanied by incidence of chemoresistance associated with treating colon cancer (Mohandas and Desai 1999; Mohandas 2011).

Mangroves are well known for the rich diversity of flora and fauna it harbors (Betoni et al. 2006; Lewis and Ausubel 2006). The Sundarbans in India are one of the largest mangrove forests in the world and are a huge reserve of medicinally important plants that have wide use in traditional folklore medicine. Excoecaria agallocha is one such mangrove plant that has been reported to have multifaceted bioactivity such as antimicrobial, antioxidant and antinociceptive properties (Kalamurthi and Selvaraj 2006). However, E. agallocha has not been much explored till date on the grounds of treating resistant Salmonella strains or for treating colon cancer.

Our investigation aims to evaluate the potential effect of E. agallocha extract on a clinical isolate of a multidrug resistant Salmonella typhi strain and to simultaneously study the effect of the plant extract on DLD-1 human colon cancer cell line in vitro.

Methods
Plant collection and preservation
Fresh leaves of Excoecaria agallocha were collected from Bali Island (procuring permission and approval of local and government administration) of the Indian Sundarbans (between 21° 013’ N and 22° 040’ N latitude and 88° 003’ E and 89° 007’ E longitude). The plant samples were washed with distilled water and stored at 4 °C after collection and utilized within 7 days for extract preparation (Sett et al. 2014).

Preparation of E. agallocha leaf extract
The leaves of E. agallocha were washed under running tap water, dried in sunlight for about 3–4 days and then grounded to fine a powder and stored in airtight bottles. 25 g of the powdered plant material was added to 50 mL ethanol (95%) and kept on an orbital shaker for 48 h at room temperature. Extracts was filtered with a membrane filter and dried using a rotary vacuum evaporator (RotaVap, Superfit, PBV-7D). The dried extract was collected, freeze-dried and stored at 4 °C till further experiments were performed (Patra et al. 2012).

Isolation of fractions from crude plant extract by silica gel column chromatography
Freeze-dried extract of the leaf sample of E. agallocha was dissolved in water (1:10 w/v) and adsorbed onto silica gel (purchased from Sigma Aldrich) by triturating in a mortar and left for about 10 h to dry. Silica gel (100–200 mesh) was used as stationary phase, and a glass column was used that had a dimension of 25 cm × 2 cm (NR Scientific, Kolkata). Packing of the column with silica gel was done by wet slurry method. While packing, a padding of cotton was placed at the bottom of the column. Slurry was made with the required amount of stationary phase (silica gel) in the solvent of slowest polarity (n-hexane) and gradually poured into the column to form a bed of silica. The extract was mixed with the silica gel (1:3 ratio). It was then poured on to the top of the packed silica gel, a layer of cotton was covered again, and the mixture was allowed to percolate. The column was then eluted with n-butanol:acetic acid:water (4:1:1), as this solvent system has been standardized for ethanolic E. agallocha extracts in previous studies as well (Patra et al. 2012). Twelve fractions were initially collected, each fraction was subjected to Thin Layer Chromatography (TLC), and their retention factor (Rf) values noted. Silica gel 60 F254 plate (Merck) of uniform thickness of 0.2 mm was used a stationary phase (Padmaja and Mundekad 2014). 10 µL of the fraction was applied on the TLC plate and developed in the solvent system in a closed glass chamber to a height of about 8 cm. The plate was sprayed with silver nitrate solution, and the Rf values of each band were recorded according to the formula:
High resolution liquid chromatography–mass spectrometry (HR LC–MS) analysis of plant fraction

The bioactive fraction selected by TLC analysis was subjected to High Resolution Liquid Chromatography and Mass Spectroscopy (HR–LCMS) for characterization and identification of the bioactive compound present in the fraction. HR–LCMS of the fraction was carried out in Sophisticated Analytical Instrument Facility (SAIF), Powai, IIT Bombay. [Instrument specification: Agilent Technologies, USA; Model: 1290 Infinity UHPLC system, 6550 iFunnel Q-TOFs; Mass range: 50–3200 amu; Column details: Syncronis C18 100 × 2.1, particle size 1.7µ; acquisition time-30 min; flow rate 0.3 mL/min]. The plant fraction was lyophilized till further use. For antibacterial and anticancer assays, dilutions were done with double distilled water from the freeze-dried extract to obtain various concentrations of the plant extract fraction.

Maintenance of Salmonella typhi cell culture

A multidrug resistant (MDR) strain of Salmonella typhi was detected in the blood sample of a patient at Peerless Hospital and B. K. Roy Research Centre, Kolkata, India, and was identified in the VITEK-2 automated system in the hospital. The strain was observed to be resistant against the antibacterial drugs ampicillin, aztreonam, cefotaxime, ceftazidime, chloramphenicol and imipenem determined by the universal criteria of Clinical and Laboratory Standards Institute (CLSI, 2018). Fresh subcultures were made in the preceding day of the experiment from the stock cultures maintained in the laboratory.

In vitro antimicrobial testing of the plant fraction and determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) assay was done by serial dilution method (Perumal et al. 2012), using 96 well plates and plate reader (Erba Lisa Scan II Transasia Mannheim, Germany). 100 µL of Mueller–Hinton broth (HiMedia, India) was dispensed in all the wells of the plate. 100 µL of stock concentration (1 mg/mL) of the extract fraction (made from the freeze dried-extract) was added to the first well of each column. Serial double dilution was done till the eighth well. Finally, 10 µL of 0.5 McFarland opacity culture suspension was added to each well of the plate. The plate was then gently shaken to mix the contents properly and immediately a baseline absorbance reading at 620 nm was taken. Then, the plates were kept for incubation for 16–18 h at 37 °C, and another absorbance reading at 620 nm was recorded.

Effect of extract on membrane of bacteria by propidium iodide staining method

Propidium iodide (PI) staining method was followed for studying any membrane damage in the test bacteria (Riccardi and Nicoletti 2006). The bacterial strain was inoculated in Luria Bertani (4 mL) broth and treated with the MIC of plant extract (4 mL) and incubated overnight at 37 °C. After incubation, 10 µL of the culture was treated with 10 µL of Propidium iodide (stock concentration of PI used was 10 µg/mL in PBS stored at 4 °C), and the suspension was taken on a slide and observed under a fluorescence microscope (EPI Fluorescence Microscope N400 YX2C with Digital Camera P7800 & Photo Relay lens).

Observing effect on Omp protein of S. typhi by sodium dodecyl polyacrylamide gel electrophoresis (SDS–PAGE) protein profiling

Total protein isolation

Whole cell lysates of Salmonella for Sodium Dodecyl Polyacrylamide Gel Electrophoresis (SDS–PAGE) analysis were prepared (as described by Aksakal 2010). Briefly, one colony of the resistant strain of S. typhi with and without treatment of plant extract (at MIC) was transferred from MacConkey agar plate to100 mL of Tryptic Soy Broth and incubated overnight at 37 °C. Subsequently, the broth culture was centrifuged at 15,000 rpm for 15 min at 4 °C. The sediment was resuspended in 10 mL of Phosphate Buffer Solution (PBS, pH 7.2). One mL of the suspension was transferred into 1.5 mL microcentrifuge tubes and centrifuged at 15,000 rpm for 15 min at 4 °C. The sediment was suspended in 10 µL of 10% SDS and an equal volume of loading buffer [0.125 M Tris (hydroxymethyl) aminomethane, 4% SDS, 10% 2-mercaptoethanol, 0.2% bromophenol blue] was added. After vigorous shaking by vortex, the prepared samples were boiled for 10 min at 100 °C, centrifuged for 1 min (15,000 rpm at 20 °C), and the supernatants were stored at –20 °C until use. SDS–PAGE was carried out, and the gel was analyzed for any protein followed by analysis of the results obtained.

SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

Protein samples were heated with 1X SDS loading buffer (50 mM Tris-Cl; pH-6.8), 2.5% β-mercaptoethanol, 2%
SDS, 0.1% bromphenol blue and 10% glycerol at 95°C for 10 min to bring out the extract. The samples were then centrifuged at 10,000 rpm for 2 min at 4°C and then resolved by SDS-PAGE. Samples were run in SDS-gel running buffer using an electrophoresis cell (Aksakal 2010). SDS-PAGE was run at 40 mA and 280 V to resolve the components (BIORAD gel apparatus was used). The first well was loaded with the molecular weight marker (Thermo Fisher). The gel is viewed in the Ultraviolet (UV) gel documentation machine (Vilber Lourmat, Mega Bio-Print, 1100/20 M, France).

**Effect of plant extract on susceptibility of S. typhi to antibacterial drugs**
The *S. typhi* was treated with the plant extract (with its MIC) for 24 h, and the antibacterial drug susceptibility was screened using agar well diffusion method described by Bauer et al. (1966) using antibacterial discs (HiMedia) of ampicillin (30 µg), aztreonam (30 µg), cefotaxime (10 µg), ceftazidime (30 µg), chloramphenicol (50 µg) and imipenem (10 µg). If any inhibition was observed, the corresponding MIC of the antibacterial drugs was determined using the serial dilution method described above in Sect. 2.6.

**Maintenance of human cervical cancer cell lines**
Human adenocarcinoma (DLD-1) cells were maintained in RPMI-1640 medium (Invitrogen) containing 10% Fetal Bovine Serum (Sigma) and 1× antibiotic–antimycotic (Invitrogen) at 37°C and pH 7.4 in a 5% CO2 humidified incubator. Peripheral blood mononuclear cells (PBMCs), used to study the selective cytotoxic potential of the plant extract, were maintained similarly in Roswell Park Memorial Institute-1640 medium (RPMI-1640) (HiMedia). DLD SCAT3-NLS expressing cells stable cells stable cells were developed by Dr T.R Santhosh Kumar's Lab, Rajiv Gandhi Centre for Biotechnology (RGCB), Kerala for cellular imaging analysis by fluorescence microscopy.

**In vitro antiproliferative activity of the bioactive plant fraction on human colon cancer cell line by MTT assay**
The evaluation of cytotoxicity of the purified plant extract fraction of *E. agallocha* was done on human colon cancer cell line (DLD-1) and healthy peripheral mononuclear cells (PBMC) by using MTT assay (Hackenkamp et al. 1999). Briefly, the cells were cultured in 96-well plates at density of 2.5 × 10^4 cells per well in the presence of the plant extract at various concentrations (0–30 µg/mL). After incubation for 48 h, MTT dissolved in PBS was added to each well at a final concentration of 5 mg/mL and then incubated at 37°C and 5% CO2 for 2 h. The water-insoluble dark blue formazan crystals that formed during MTT cleavage in actively metabolizing cells were dissolved in DMSO. The optical density was read by a microplate reader at a wavelength of 570 nm. Results were expressed as the mean of three replicates as a percentage of control (taken as 100%). The extent of cytotoxicity was defined as the relative reduction of optical density (OD), which correlated to the number of viable cells in relation to cell control (100%). The cell viability was plotted in a graph, and the IC50 was calculated accordingly to determine the optimum dosage of the extract for further studies.

**Study of apoptosis by cellular imaging analysis**
The DLD SCATS NLS stable cells were incubated with the purified plant extract in three different concentrations (IC50 value and two concentrations above and below the IC50) for 24 h in 5% CO2 at 37°C. After 24 h incubations, cells were imaged viewed under Inverted fluorescent microscope EclipseTi2 (Nikon, Japan) equipped with Spectra X light source from Lumencore. DLD SCAT3 NLS cells were imaged using excitation at 440 nm, and two emissions were collected at 450–470 and 520–550 nm using specific filters placed on the external wheel. The images were captured with an EMCCD camera (AndorIXON 897) using NIS element software (Nikon) for CFP/YFP ratio determination.

**Caspase-3 activity by colorimetric assay**
Caspase-3 Colorimetric assay kit (BioVision K106-25) was used, and the protocol described in the kit. Caspase-3/CPP32 Colorimetric Assay Kit provides a simple and convenient means for assaying the activity of caspases that recognize the sequence DEVD. The assay is based on spectrophotometric detection of the chromophore p-nitroaniline (pNA) after cleavage from the labeled substrate DEVD-pNA. The pNA light emission can be quantified using a spectrophotometer (Mecasys, Optizen) at 405-nm. Comparison of the absorbance of pNA from an apoptotic sample, induced with the plant extract treatment with different concentrations for 24 h, with an uninduced control allows determination of the fold increase in caspase activity.

**Western blot analysis**
Western blot analysis was done using a kit-based assay (Fast Western Blot Kit, Thermo Fisher Scientific). DLD-1 cells (1.0 × 10^6 cells/60), without treatment and with plant extract treatment for 24 h, were detached, washed in ice-cold cold PBS, and suspended in 100 1 L of lysis buffer. The suspension was kept on ice for 20 min and then centrifuged at 5000 rpm for 20 min at 4°C. The protein extracts were resuspended in sample buffer, and this
mixture was boiled for about 5 min. 50 µg of the proteins were loaded into each lane, resolved on a 10% Sodium dodecyl-sulfate (SDS) polyacrylamide gel (PAGE) and then transferred to nitrocellulose membrane (Saha et al. 2015) for Western blot using the required antibody against MMP-7 was used. Protein of interest was visualized by using Trans-Blot Turbo Transfer system (Bio-Rad). β-actin was used as control.

Statistical analysis
All experiments are expressed as mean ± standard deviation from at least three independent experiments performed in triplicates. One way ANOVA was used in Microsoft Excel (Version 2016) using the data analysis tool. Results with \( p < 0.05 \) were considered to be statistically significant.

Results
Isolation of bioactive fraction and characterization by LC–MS
Three fractions were obtained after pooling the eluted fractions from column chromatography based on the TLC analysis. Out of the three fractions, two fractions yielded one spot each while the third fraction yielded three distinct bands on developing with silver nitrate solution. The Rf values of the spots were calculated as 0.14, 0.127 and 0.09. The observation was indicative of the fact that this fraction might contain important phytochemical compounds such as phenolic derivatives and glycosides according to literature studies. HR-LCMS results of the fraction indicated the presence of two major compounds at 280 nm scan (Fig. 1) along with a few more compounds in comparatively lower abundance. The bioactive compounds identified were—Bergenin and Hexanoylglycine. Out of these compounds identified, Bergenin (328.08 g/mol), a trihydroxybenzoic acid glycoside, was present in the highest abundance and its molecular formula and mass were determined by LC–MS (Table 1).

Antimicrobial activity screening assay and determination of minimum inhibitory concentration (MIC)
The antimicrobial activity of the plant extract was screened for the concentrations 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL, 31.75 µg/mL, 15.875 µg/mL, 7.93 µg/mL and 3.96 µg/mL. The MIC value of the plant fraction was observed to be 15.7 ± 0.16 µg/mL (Fig. 2), and this concentration was used for further experiments.

### Table 1 List of compounds characterized by HR LCMS in *E. agallocha* extract

| Sl. no | Compound              | Molecular weight (g/mol) | Formula      | m/z value        | Relative intensity (%) |
|--------|-----------------------|--------------------------|--------------|------------------|------------------------|
| 1      | Bergenin              | 328.08                   | C_{14}H_{16}O_{9} | 333.0595        | 127.34                 |
| 2      | Hexanoylglycine       | 173.10                   | C_{8}H_{15}NO_{3} | 156.1018        | 31.13                  |
| 3      | Tyramine              | 137.08                   | C_{8}H_{11}NO  | 11.21            |                        |
| 4      | 6-Phosphogluconic acid| 276.02                   | C_{6}H_{13}O_{10}P |                | 38.33                  |

*m/z* mass-to-charge ratio
Effect on bacterial membrane by plant extract using propidium iodide staining

It is well known that propidium iodide (PI) may intercalate into double-stranded nucleic acids and fluoresces. It is excluded by viable cells but they can penetrate cell membranes of dead cells. Thus, PI staining is included in immunofluorescent staining protocols to identify dead cells. PI is excited at 488 nm and emits at a maximum wavelength of 617 nm due to Stoke's shift phenomenon. In our investigation, PI staining led to the inference that outer cell membrane of *Salmonella* was disrupted on treatment with the plant extract (with MIC concentration) (Fig. 3) as is evident by the increased number of PI-stained cells because PI
stains only those cells whose membrane has been damaged and thus, the stain crosses it and stains the DNA within. Thus, it could be affirmed that the extract inhibited the growth of the bacteria, and the plausible mode of action of the extract might be interfering with the membrane integrity of the bacteria.

**Observing effect on Omp protein of *S. typhi* by SDS-PAGE protein profiling.**

The total protein was isolated and was analyzed by SDS PAGE, and the bands were studied. The resistant strain shows the presence of a 33 kDa band in Lane 1 (Fig. 4) and according to literature studies, it is thought to confer antibacterial resistance by increasing the membrane integrity and escalating drug efflux. When treated with the MIC of the plant fraction for 24 h, this protein band was absent, indicating that the bioactive plant fraction is disrupting the membrane integrity by down regulation of OmpA protein expression.

**Effect of plant extract on susceptibility to antibacterial drugs**

It was interesting to note that on treating the *S. typhi* with the MIC of the plant extract, the bacteria became intermediately susceptible to a few antibacterial drugs as observed by MIC values (Table 2). The interpretations were made according to CLSI standards wherein a bacterial strain is said to be “susceptible-dose dependent” when it is sensitive to a given antibacterial drug and its growth is inhibited with a probability of therapeutic success but multiple approved dosing options have to be checked while “intermediate sensitivity” means that the bacteria is inhibited in vitro by a concentration of the antibacterial drug that is associated with an uncertain therapeutic effect. Though none of the strains became absolutely sensitive to the antibacterial drugs but became susceptible to a certain extent depending upon the dose of the extract and the antibacterial drug taken together. The sensitivity to antibacterial drugs might be conferred by the disruption of membrane integrity, and OmpA protein that might have led to increased diffusion of drugs across the bacterial cell membrane.

**Table 2** Change in MIC of antibacterial drugs against *S. typhi* strain treated with plant extract

| Sl. no | Antibacterial drug | MIC (in µg/mL) of *S. typhi* Without plant extract treatment | With plant extract treatment |
|--------|--------------------|-------------------------------------------------------------|------------------------------|
| 1      | Ampicillin         | > 32 (R)                                                   | 30.25 ± 1.40 (SSD)          |
| 2      | Aztreonam          | > 16 (R)                                                   | 14.13 ± 1.38 (SSD)          |
| 3      | Cefotaxime         | > 4 (R)                                                    | 2.43 ± 0.50 (IS)            |
| 4      | Ceftazidime        | > 16 (R)                                                   | 34 ± 29.28 (R)              |
| 5      | Chloramphenicol    | > 32 (R)                                                   | 18.28 ± 1.1 (IS)            |
| 6      | Imipenem           | > 4 (R)                                                    | 3.21 ± 0.25 (SSD)           |

*R* resistant, **SSD** susceptible-dose dependent, **IS** intermediate susceptibility

**Toxicity study against healthy mammalian cell line and cancer cell line by MTT assay**

The results of MTT assay showed a remarkable cytotoxic activity of the lant fraction against the DLD-1 cell line (Fig. 7). The results of the MTT assay indicated that the IC50 value of the plant extract fraction was about 17.99 ± 1.12 µg/mL (Fig. 5a). Moreover, the plant fraction induced a significant increase in cell proliferation when treated on healthy PBMC cells (Fig. 5b), which is indicative of its selective toxicity. Thus, this extract would not produce toxicity in normal healthy cells in the applied dosage concentration (i.e., 18 µg/mL).

**Apoptosis study by cellular imaging analysis**

Apoptosis was studied by the method described above. Control cell showed low CFP/YFP Ratio which is in accordance with the methodology (Fig. 6). 24 h treatment
of plant extract (30 µg/mL) showed increase in caspase-3 activation, which was evident because the CFP/YFP ratio gradually increased in DLD SCAT3 NLS cells on treatment with the plant extract fraction (Fig. 6). The CFP/YFP ratio in the plant fraction treated DLD1 cells increased in a dose dependent manner that leads us to the inference that the extract causes caspase-3 mediated apoptosis in the colon cancer cells (Fig. 7).

Caspase-3 activity
This assay utilizes a synthetic tetrapeptide, Asp-Glu-Val-Asp (DEVD), labeled with a colorimetric molecule, p-nitroanilide (p-NA) as substrates. The results indicate that the caspase-3 content increases in DLD-1 cells in a dose-dependent manner (Fig. 8), validating the imaging analysis observations. Induction of apoptosis by the plant extract is associated with an increase in the DEVD-dependent protease activity that ultimately leads to apoptosis of the cancer cells, as activated caspase-3 in apoptotic cells cleave the substrate into free p-NA which is determined spectrophotometrically at 405 nm.

Effect of plant extract on MMP-7
We analyzed the effect of the plant extract on MMP-7, a zinc-binding metalloproteinase that is responsible for colorectal cancer progression and metastases. Our observations indicated that there was marked decrease in the expression of MMP-7 in the DLD-1 cells in a dose-dependent manner (Fig. 9). β-actin was used as loading control.

Discussion
Incidence of antimicrobial resistance has escalated in the past years at an alarming rate, demanding the need to develop new chemotherapeutic agents from plants. Herbal medicines are gaining attention in the present scenario due to the multi-potent biological activities
Fig. 7 Mechanism of apoptosis study by Caspase-3 release using SCAT3 NLS ratiometric probe

Fig. 8 Colorimetric assay of Caspase-3 activity in DLD-1 cells (*results are statistically significant as compared to the control set)
exhibited by the phytochemicals. The Sundarbans are a huge reserve of such medicinally important plants that may be explored to combat the issues such as antimicrobial resistance and chemoresistance. *E. agallocha* was collected from the Sundarbans in West Bengal and studied for its potential against antibacterial drug resistant *Salmonella typhi* and its effect on human colon cancer cell line.

Very few research studies in India have been conducted to evaluate the antimicrobial potential of mangrove plants against *S. typhi*. One study revealed that dried leaf samples of *E. agallocha* collected from Muthupetai mangrove forest of Thiruvarur district of Tamil Nadu, India, showed good antimicrobial activity against *S. typhi* (Prakash and Sivakumar 2013). In another study, an ethanolic extract of leaves of *Sonneratia alba* collected from Chorao Island, Goa, India, showed good antimicrobial activity against *S. typhi* (Sahoo et al. 2012). The results of our investigation show that a specific fraction of *E. agallocha* extract has significant antibacterial activity against the MDRO strain of *Salmonella* under study. The MIC was determined to be $15.7 \pm 0.16 \mu g/mL$, which is quite remarkable. HR LC–MS revealed that this fraction had two major bioactive compounds namely, bergenin and hexanoylglycine. Bergenin was present in a relatively larger amount and has not been reported to be extracted from *E. agallocha* till date. PI staining gave us a qualitative idea that the extract permeabilized and disrupted the membrane of the bacteria. To further validate this finding, protein profiling and analysis were done by visualizing the different proteins in SDS PAGE. The results clearly show the absence of a 33 kDa band in the extract treated bacteria which is thought to contribute to antibacterial drug resistance. Further, on treatment with the plant extract, the bacterial strain became moderately susceptible to a few antibacterial drugs that it was resistant to earlier. The outer membrane of Gram-negative bacteria such as *S. typhi* contains an intricate framework consisting of lipopolysaccharides, phospholipids and porins (Henderson et al. 2016). This membrane also helps in inhibiting the transport of various compounds including antibacterial drugs and bile acids that are detrimental for the bacteria. (O’Shea and Moser 2008; Page et al. 2008). This characteristic of membrane often serves as an obstacle in developing novel antimicrobial drugs that target such pathogens leading to failure of treatment strategies and multidrug resistance (Lee et al. 2013). Among the various membrane proteins of *S. typhi*, outer membrane protein A (OmpA) is a non-specific porin through which only selected chemicals can pass through by passive diffusion (Sugawara and Nikaido 1992; Iyer et al. 2018). OmpA and OmpF are closely related and mutations or change in expressions of these proteins have been reported to be the contributing factors for antibacterial drug resistance in various Gram-negative bacteria. Antibacterial drugs such as β-lactams and fluoroquinolones penetrate the bacterial cell through the porin OmpF (Mach et al. 2008; Delcour 2009). Thus, the ompF mutant strains have been reported to be resistant to several β-lactam antibacterials in some Gram-negative pathogens, including *Escherichia coli* (Nikaido et al. 1983; Ziervogel and Roux 2013), *Klebsiella pneumoniae* (Sugawara et al. 2016), *Serratia marcescens* (Moya-Torres et al. 2014), *Pseudomonas aeruginosa* (Okamoto et al. 2001) and *Enterobacter aerogenes* (Bornet et al. 2000). On the other hand, mutation, downregulation or deletion of OmpA have been reported to be associated with susceptibility to a number of antibacterial drugs including β-lactams in *Acinetobacter baumannii* (Smahi et al. 2014). This feature may be attributed by the effect of OmpA that is known to maintain bacterial cell membrane integrity. Disruption of the membrane structure or impaired membrane integrity can increase the diffusion of certain antibacterial drugs. OmpA is known to affect membrane integrity through noncovalent interaction of its C-terminal periplasmic domain with peptidoglycan (Samsudin et al. 2016). The role of the C-terminal domain appears to be pivotal for the role of OmpA in antibacterial drug resistance as well as the maintenance of membrane integrity. Thus, our results are in accordance with previous findings, and the fraction obtained from *E. agallocha* extract is not only effective individually but may be used synergistically with antibacterial drugs in combination therapy to combat the problem of multidrug resistance in *S. typhi* and the infections associated with it.

Colon cancer is a major cause of cancer morbidity and mortality worldwide, especially for older patients, as colon cancer incidence increases markedly after the age of 60 years. Further, decrease in cell adhesion and proteolytic degradation of collagen by matrix metalloproteinases (MMPs) promote the invasive migration of colon cancer cells through the extracellular matrix (Adachi et al. 1999; Yoshimoto et al. 1993). Among more than 20 MMPs, matrilysin (MMP-7) appears to be one of the most important MMPs in human colon cancers, because it is highly
was studied by a FRET–based genetically encoded fluorescence caspase specific sensors. The caspase-3 recognition sequence (DEVD), was used as a linker between the FRET pair CFP and YFP with nuclear localization signal (NLS). Upon the induction of apoptosis, the activated caspase-3 cleaves the DEVD sequence resulting loss of FRET and can be visualize ratiometrically (CFP/YFP). Control cell showed low CFP/YFP ratio which is accordance with the desired results. 24 h treatment of plant extract fraction showed an increase in caspase-3 activation in a dose-dependent manner, which was evident as increased CFP/YFP ratio levels in DLD SCAT3 NLS ratiometric probe. Caspase-3 activation was confirmed by colorimetric assay that showed an increase in the level of Caspase-3 activity in a dose-dependent manner, and the results were in accordance to the previously observed events. Further, the MMP-7 protein expression was reduced in the plant extract treated DLD-1 cells in a dose-dependent manner, indicating that the proliferation and metastasis of the colon cancer cells may be inhibited by this plant extract fraction. Our results, thus show a promising anticancer effect of the *E. agallocha* extract that might lead to an inhibition of the metastasis process of the colon cancer cells by decreasing the MMP-7 expression while simultaneously increasing the Caspase-3 level that will cause apoptosis of the cancer cells. However, further studies need to be undertaken to facilitate the testing of this plant extract on other multidrug resistant pathogenic bacteria and fungi as well as on other cancer cell lines that would eventually lead to its application in animal models and human trials for developing an efficient chemotherapeutic agent.

**Conclusions**

The findings suggest that the antimicrobial and anticancer property of the *E. agallocha* extract may be due to the presence of the compound Bergenin in high abundance. *E. agallocha* extract may be exploited to obtain Bergenin that has a potential role in overcoming antibacterial resistance and in the treatment of colon cancers in human beings.

**Abbreviations**

CFP: Cyan fluorescent protein; CLSI: Clinical and laboratory standards institute; DEVD: Asp-Glu-Val-Asp; DMSO: Dimethyl sulfoxide; FRET: Fluorescence resonance energy transfer; HR LCMS: High resolution liquid chromatography mass spectroscopy; IC50: Half-maximal inhibitory concentration; MMP-7: Metalloproteinase-7; MTI: (4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NLS: Nuclear localization sequence; OD: Optical density; OmmA: Outer membrane protein A; PBMC: Peripheral blood mononuclear cells; PBS: Phosphate buffer saline; PI: Propidium iodide; p-NP: Para-nitroanilide; R: Retardation factor; RPMI 1640: Roswell Park Memorial Institute-1640 medium; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; TLC: Thin layer chromatography; UV: Ultraviolet; YFP: Yellow fluorescent protein; WHO: World Health Organization.

**Acknowledgements**

The authors sincerely thank Rev. Dr. Dominic Savio, S.J., Principal, St. Xavier’s College (Autonomous), Kolkata, for providing the laboratory facilities required for conducting the research. The authors acknowledge the support and cooperation from the Department of Microbiology, St. Xavier’s College (Autonomous), Kolkata and Peerless Hospital & B K Roy Research Centre, Kolkata, for providing infrastructure and lab facilities to execute the antimicrobial assays. We wish to thank Dr. Saurav Seth, Department of Marine Science, University of Calcutta, for helping us in the procurement and identification of the plant samples. Our sincere thanks to the team at Sophisticated Analytical Instrument Facility (SAIF), IIT-Bombay, for providing the HR LC-MS facility. We convey our sincere gratitude to Dr. T.R. Santhosh Kumar and his team at Rajiv Gandhi Centre for Biotechnology, Kerala for providing the cellular imaging analysis facility. We also acknowledge the support gained from CCRH, Kolkata for conducting the MTT assay and protein expression studies.

**Authors’ contributions**

TS performed the experiments, data analysis and composed the manuscript. SD and AKM conceptualized, designed the work and corrected the primary manuscript. All authors read and approved the final manuscript.

**Funding**

This research was supported by WB-DBT (Department of Biotechnology, Government of West Bengal) under the sanctioned project: Sanc. No. 541(Sanc)-BT/ST/P&S&T/0G-45/2017. Grant was provided from this funding body for purchase of chemicals, reagents, apparatus, glasswares and for availing instrumentation facilities (outsourcing).

**Availability of data and materials**

All data generated and analyzed during the experiments that support the findings are included within the article.

**Declarations**

**Ethics approval and consent to participate**

The guidelines followed for blood isolation (for PBMC used in MTT assay) from healthy volunteer donors have been approved by the Institutional Ethical Committee. Consent to participate is not applicable in our investigation.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interest.

**Received:** 29 September 2021  **Accepted:** 18 November 2021  **Published online:** 04 December 2021
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