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Antibacterial and cell envelope damaging properties of different solvent extracts of *Rhus chinensis* Mill against *E. coli* and *Staphylococcus aureus*

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Rhus chinensis mill., is a member of the Anacardiaceae family and it is known for treating diarrhoea, dysentery, food poisoning and gastritis. *R. chinensis* contains a wide array of substances that can cure various infectious diseases. These substances are biologically active components also known as secondary metabolites or phytochemicals, which help to combat numerous diseases in the modern era. The decoction of the fruits of *R. chinensis* is useful for the treatment of dysentery, diarrhoea, indigestion and exhibit potential anticancer, antiviral, antimicrobial, antidiarrhoeal and anti-inflammatory activities.

Extracts of aqueous, ethyl acetate and methanol were tested against microorganisms and demonstrated antibacterial activity. The sample preparation for the SEM study was done according to the method described by Borthakur and Joshi. At the minimum inhibitory concentration (MIC) and concentration of 400 mg/mL the test bacteria *E. coli* and *Staphylococcus aureus* were treated with methanol extract and kept at 37ºC overnight for incubation. After incubation, the bacterial cultures were centrifuged for 20 min at 4ºC at 1000 rpm, then washed three times with 0.1M sodium cacodylate buffer (pH 7.3) and three times with distilled water by centrifuging for 3 min at 3000 rpm. *R. chinensis* exhibited significant antibacterial activity. The result of time kill assay of *E. coli* treated with methanol extract exhibited a twofold log reduction in the bacterial count at 90 min for MIC (12.5 mg/mL) and 60 min for 2 x MIC (25 mg/mL). The bacterial cells treated with the methanolic extract of *R. chinensis* revealed the leakage of cellular materials which exhibited absorbance at the wavelengths of 260 nm and 280 nm.

Keywords: Antibacterial, Cellular leakage, *R. chinensis* Mill, Scanning electron microscope, Time kill assay
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Antibiotic resistant bacteria have become prevalent due to the indiscriminate use of antibiotics[1]. Therefore the focus has shifted towards the plant based medicine, which can be a good alternative for antimicrobials[2]. The medicinal plants contain a plethora of natural constituents that can cure various infectious diseases. These substances are biologically active components also known as secondary metabolites or phytochemicals, which help to combat numerous diseases in the modern era[3]. Phytochemicals such as phenolic compounds, tannins, saponin, aldehyde and flavonoids are the major active principles for many plant-based drugs[4].

R. chinensis Mill locally known in Sikkim as *Bhakiamilo*[5] is adioecious shrub [6] belonging to the family Anacardiaceae. The genus *Rhus* commonly known as Sumac[7] contains 250 species distributed in tropical, subtropical and temperate regions of Asia[8]. Sikkim is a north eastern state of India and is a biodiversity hotspot region[9]. In Sikkim, *R. chinensis* is found in the dry forest slopes between 915 m to 2745 m with the fruit season from October to December. The fruits are edible and infusion of the fruits is used to treat dysentery, diarrhoea and indigestion[10]. The Apatani tribes of Arunachal Pradesh use the fruit of *R. chinensis* during blood dysentery[11]. A concoction of the fruit juice, water andraw egg, is used by the Lepcha tribe of the Dzongu valley, North Sikkim to treat diarrhea and dysentery. The fruit is also used as a food preservative[9]. *R. chinensis* have been reported to exhibit potential anticancer[12] antiviral[13], antimicrobial[14], antidiarrhoeal[15] and anti-inflammatory[16] activities. The phenolic compounds gallic acid and methyl gallate are present in *R. chinensis* Mill. The roots and leaves of this plant have been reported to be rich sources of various bioactive compounds[17]. The aqueous extract of *R. chinensis* has shown activity against *Helicobacter pylori*[18]. The hydroalcoholic leaf extract of *R. chinensis* mill has potent wound healing properties[19]. Therefore,

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the present study evaluates the antibacterial and cellular leakage properties of different solvent extracts of *R. chinensis* mill against *E. coli and S. aureus.*

Methodology

All the chemical reagents and culture media used in the study were procured from Sigma-Aldrich, USA, Merck, Germany and HiMedia, India.

Sampling and description of plant specimens

R. chinensis Mill was collected from Amba, East Sikkim, India, (27.19232 N and 88.6796 E). The plant sample was taxonomically identified and the voucher specimen with the accession number 09736 was deposited at the herbarium of the Plant Taxonomy Division, Department of Botany, University of North Bengal, Siliguri, West Bengal, India.

Preparation of plant extracts

The dried fruit of *R. chinensis* Mill was pulverized into a fine powder using Waring blender (Cole Parmer, RZ-04245-21). The powdered material (10 g) was separately processed for 24 h using 100 mL of distilled water, ethyl acetate solvent and methanol solvent to prepare the aqueous, ethyl acetate and methanol extracts respectively. The extracts (aqueous, ethyl acetate and methanol) were subjected to Rotary evaporator treatment (Bhuchi, Switzerland, R-3) and kept for further use in sterilized vials at 4ºC[20].

Test microorganisms

The test microorganisms were procured from the Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh India. For the present study, *S. aureus* (MTCC-7443) and *E. coli* (MTCC-3384) were used as the test bacteria.

Phytochemical analysis

The aqueous extract, ethyl acetate solvent extract and methanol solvent extract of the plant sample were subjected to standard phytochemical analyses to examine the phytoconstituents namely phenol, flavonoid, tannin, saponin, steroid, anthocyanin, alkaloid, glycoside, carbohydrate, protein and fat[21,22,23].

Antibacterial Activity

The antibacterial activity of aqueous, ethyl acetate and methanol extracts of *R. chinensis* Mill was estimated by using agar well diffusion technique[24]. The aqueous, ethyl acetate solvent and methanol solvent extracts were dissolved using 0.25% dimethyl sulphoxide (DMSO) to obtain various concentrations (20 mg/mL, 100 mg/mL, 200 mg/mL and 400 mg/mL) of the solvent extracts. Gentamicin (0.1 mg/mL) was kept as the positive control[25] and DMSO (0.25%) was kept as the negative control. The antimicrobial activity was determined by measuring the diameter of zone of inhibition including the well diameter of 8 mm. All the experiments were conducted in triplicate and the data shown are the mean value±SD values. For determination of minimum inhibitory concentration (MIC), double fold dilution of extracts were made in the range of 400–0.048 mg/mL as described by Wiegand *et a.l*[26]. For the evaluation of the Minimum Bactericidal Concentration, 100 µL of broth cultures that showed no bacterial growth was reinoculated on Mueller Hinton Agar plates, then was incubated for 24 h at 37ºC as defined by Heredia *et al*[27].

Total phenolic, flavonoid and tannic acid content

Folin–Ciocalteu method as defined by Lister and Wilson[28], Aluminum chloride colorimetric method as defined by Chang *et al.*[29] and Folin-Denis method as described by Polshethiwar and Ganjjiwale[30] respectively determined the total phenolic, flavonoid and tannic acid contents present in the various solvent extracts. The total phenolic, flavonoid and tannic acid contents were expressed as µg of Gallic acid equivalent (GAE), µg of Rutin equivalent (RE) and µg of Tannic acid equivalent (TAE) respectively.

Time Kill assay

The rate of killing of bacterial cells by the crude methanolic extract was performed using the method adapted from Saritha *et al.*[31]. Dilution of the overnight bacterial culture was done with a 10 mM sodium phosphate buffer (pH 7.4) to maintain the OD of bacterial cells ranging from 0.08-0.13 at 625 nm. To approximately 2×10^6 CFU/mL bacterial culture the extracts at concentrations of ½ x MIC, MIC and 2 x MIC was added and incubated for 0–120 min at 37°C. The assay mixtures were diluted serially with 10 mM sodium phosphate buffer (pH 7.4) after incubation. 100 µL of each had been plated on an agar plate and incubated for 24 h at 37°C. The plant extract treated bacterial colonies and untreated colonies were enumerated and plotted as CFU/mL.

Cellular Leakage assay

The overnight bacterial culture was centrifuged for 10 min at 10,000 rpm. The pellets obtained after

centrifugation were resuspended in sterile 0.85% sodium chloride solution. Suspensions were then adjusted to achieve OD of 1.5 at 600 nm. 1 mL of the aqueous, ethyl acetate solvent and methanol solvent extracts at the concentrations of the MIC and 400 mg/mL were added to separate test tubes containing 1 mL of bacterial suspension. The suspension was incubated at 0, 30, 60, 90 and 120 min. The bacterial culture was again centrifuged for 10 min at 10,000 rpm after incubation and the supernatant was transferred to a sterile vial. The supernatant absorbance was measured at 260 nm and 280 nm with UV-VIS spectrophotometer. The net cellular leakage value represents the calculated values after subtracting the absorbance value of untreated bacterial cells and the absorbance value of the plant extract from the absorbance value of the treated bacterial cells at 260 nm and 280 nm[32]. The amount of protein in the supernatant was measured using Bradford reagent. The standard protein used was Bovine serum albumin (BSA)[33,34].

Scanning Electron Microscope (SEM) study

The sample preparation for the SEM study was done according to the method described by Borthakur and Joshi[35]. At the MIC and at the concentration of 400 mg/mL the test bacteria *E. coli* and *S. aureus* were treated with methanolic extract and kept at 37°C for overnight incubation. After incubation, the bacterial cultures were centrifuged for 20 min at 4°C at 1000 rpm, then washed thrice with 0.1M sodium cacodylate buffer (pH 7.3) and 3 times with distilled water by centrifuging for 3 min at 3000 rpm. The cells were then fixed with 3% glutaraldehyde and kept overnight at 4°C. After removal of the glutaraldehyde, the bacterial pellets were washed three times with 0.1 M sodium cacodylate buffer at 1000 rpm for 10 min each followed by one wash with distilled water. Then the samples were mounted in the cover slip. Finally, the samples were sputter coated with gold palladium and scanned under Scanning Electron Microscope (JSM-6360; Jeol, Peabody, Massachusetts, USA) with a thin layer of gold-palladium.

Statistical Analysis

The statistical analysis was done using a two-way analysis of variance (ANOVA), using GraphPad Prism version 5.01 (San Diego, USA). A p-value <0.05 has been considered as statistically significant.

Results and discussion

Plant phytochemicals have been reported to possess antioxidant properties, hormonal activity, enzymes stimulation, DNA replication intervention and antibacterial properties[36,37]. The present study has revealed various phytochemicals in the different solvent extracts of *R. chinensis* mill. The phytochemical analyses of aqueous, ethyl acetate solvent and methanol solvent extracts of *R. chinensis* revealed the presence of tannins, flavonoid, anthocyanin, alkaloid, phenol, protein, glycosides and steroids. Phytochemicals such as phenolic compounds, tannins, saponin, aldehyde and flavonoid are the major active principles for many plant based drugs[4].

All of these phytochemicals are known to possess antimicrobial activities [38]. The antibacterial activity was determined by the method of well diffusion. The plant extract exhibiting the diameter of the inhibition zone ≥10 mm was considered to have active antimicrobial properties[39]. The present study revealed significant antibacterial activity in the aqueous, ethyl acetate solvent extracts and methanol solvent extract of *R. chinensis*. The plant extracts exhibited inhibitory effect on the bacterial growth but showed variation in efficacy of the growth inhibiting property[40]. The inhibitory effect of the various solvent (aqueous, ethyl acetate and methanol) extracts of *R. chinensis* mill was assessed against *E. coli* and *S. aureus.* The methanol extract exhibited growth inhibitory effect for both the test microorganisms at the lower concentration of 20 mg/mL. The ethyl acetate extract inhibited only *S. aureus* while the aqueous extract did not exhibit any growth inhibiting activity for the test bacteria. All the solvent extracts of *R. chinensis* showed an inhibition zone at a higher concentration of 400 mg/mL (Fig. 1)

The methanol extract was effective in inhibiting the growth of test microorganisms as compared to the ethyl acetate and aqueous extracts among the three solvents used. This was possibly because a large number of phytochemicals are extracted in the methanol solvent[39]. The variability in the growth inhibiting efficacy of the extract against various microorganisms depends on the chemical composition of the extracts and permeability of the bacterial cell membrane for the chemicals and their metabolism[41]. The less active extract which can be more diffusible can exhibit a larger inhibition zone than the more active extract that might be non-diffusible[42]. The

methanol and ethyl acetate extracts exhibited the Minimum Inhibitory Concentration at 12.5 mg/mL against both *E. coli* and *S. aureus*. For the methanol extract, the Minimum Bactericidal Concentration was 25 mg/mL for *E. coli* and 50 mg/mL for *S. aureus* whereas the ethyl acetate extract showed MBCvalue of 25 mg/mL against both *E. coli* and *S. aureus* (Table 1).

The flavonoid, polyphenol and tannin compounds have been reported to inhibit nucleic acid biosynthesis, enzyme inhibition, membrane disruption[39]. In this study, the methanolic and ethyl acetate solvent extracts exhibited a higher content of tannin followed by phenolic compound and flavonoid whereas the aqueous extract exhibited a higher content of the flavonoid (Table 2). These phytochemical compounds could account for the antibacterial activity of various solvent extracts. Therefore, based on the diameter of the inhibition zone and MIC values displayed by various solvent extracts against *E. coli* and *S. aureus*, the methanol extract of *R. chinensis* was selected to determine the possible mode of antibacterial action by means of time-kill assay, cellular leakage assay and scanning electron microscope study.

Time-kill assay gives information on the pharmacodynamics of the substances being studied[43]as to whether it affects the membrane integrity (instantaneous) or affects the cellular process (time-dependent)[31]. The study was conducted to determine the kinetics of bacterial killing at different concentrations of the plant extracts. There was a significant ($p<0.001$) time and concentration-dependent decrease in the bacterial count when treated with the methanolic extract of *R. chinensis*. This could be due to the various physiological factors inside the cell[31]. The result of time kill assay of *E. coli* treated with methanol extract exhibited a twofold log reduction in the bacterial count at 90 min for MIC (12.5 mg/mL) and 60 min for 2 x MIC (25 mg/mL) (Fig. 2). Similarly, for *S. aureus* two fold log decrease in the bacterial counts were seen for MIC (12.5 mg/mL) and 2x MIC (25 mg/mL) at 90 min and 30 min respectively (Fig. 3).

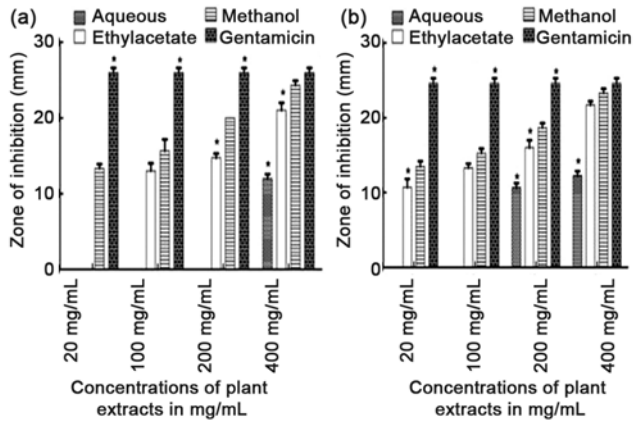

Fig. 1 — Zone of inhibition exhibited by the aqueous, ethyl acetate and methanol extracts of *R. chinensis* at the concentrations of 20, 100, 200 and 400 mg/mL against A. *E. coli* and B. *Staphylococcus aureus*. $p<0.001$ represents significant difference in the antibacterial activity of the * methanol extract against aqueous extract, ethyl acetate extract and Gentamicin

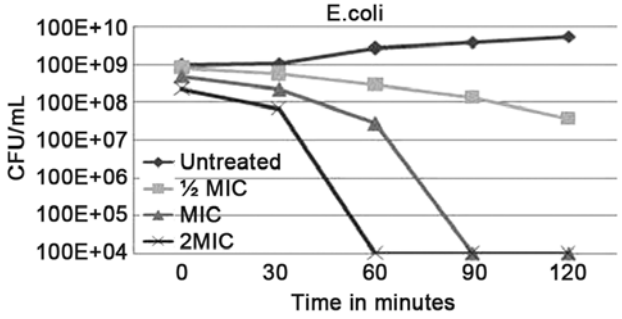

Fig. 2 — Time-kill curve of *E. coli* after treatment with the methanol extract of *R. chinensis* at concentrations of ½ x MIC, MIC, 2 x MIC at different time intervals. The values shown are mean±SD

Table 1 — MIC, MBC and the MIC index of the different solvent extracts of *R. chinensis* against *E. coli* and *Staphylococcus aureus*

Solvent Extract	Organisms	MIC (mg/mL/)	MBC (mg/mL)	MIC Index
Methanol extract	*E. coli*	**12.5**	**25**	**2**
	Staphylococcus aureus	**12.5**	**50**	**4**
Ethyl acetate extract	*E. coli*	**12.5**	**25**	**2**
	Staphylococcus aureus	**12.5**	**25**	**2**
Aqueous extract	*E. coli*	**50**	**200**	**4**
	Staphylococcus aureus	**50**	**200**	**4**

Table 2 — The total phenolic, flavonoid and tannic acid contents in the different extracts of *R. chinensis* Mill in µg GAE, µg RE and µg TAE respectively

Solvent extract	Total phenolic content (µg GAE)	Total flavonoid content (µg RE)	Total tannic acid content (µg TAE)
Aqueous extract	1.2 ± 0.05	9.57± 0.51	3.41±0.01
Ethyl acetate extract	11.7±0.058	3.87±0.06	18.98±0.06
Methanol extract	28.4±0.004	10.77±0.06	54±0.28

GAE- Gallic acid equivalent, RE – Rutin equivalent, TAE – Tannic acid equivalent

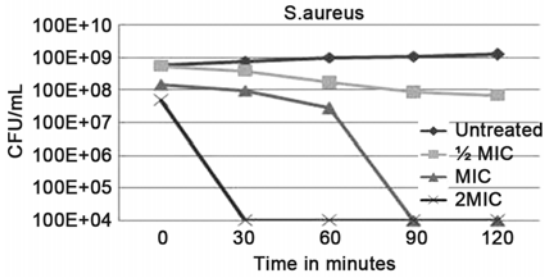

Fig. 3 — Time-kill curve of *Staphylococcus aureus* after treatment with the methanol extract of *R. chinensis* at concentrations of ½ x MIC, MIC and 2 x MIC at different time intervals. The values shown are mean±SD

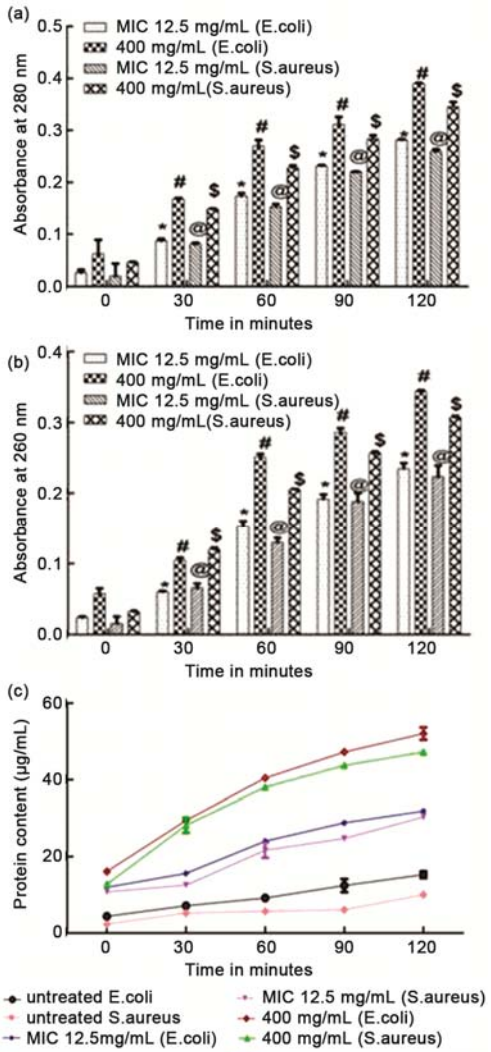

Fig. 4 — A. Absorbance at 260 nm, (B) 280 nm, (C) protein content of supernatant from *E. coli* and *Staphylococcus aureu*s suspensions of control and after treatment with the methanol extract of *R. chinensis* at the concentrations of 400 mg/mL and MIC measured at different time intervals. $p<0.05$ represent significant changes at different time intervals at the respective concentrations- * MIC for *E. coli*, # 400 mg/mL for *E. coli*, @ MIC for *Staphylococcus aureus*, $ 400 mg/mL for *Staphylococcus aureus*

The various modes of antibacterial action include damage of the intracellular membrane, interference with protein complexes (ATPases and others), secretion of lipopolysaccharides, alteration in the proton motive force with release of ions, coagulation of the cellular components and enzyme synthesis inhibition[44]. The cellular leakage assay revealed a significant increase in the release of cellular contents from the supernatant of both *E. coli* and *S. aureus* suspensions as time and concentration increased when treated with methanol extract of *R. chinensis* at the MIC and at 400 mg/mL concentrations. Leakage of cellular materials absorbing at 280 nm was found to be more pronounced than at 260 nm at the same exposure time. It has been reported that the various chemical compounds present in the crude extract affects multiple sites on the bacterial cells, there by leaching out various intracellular materials[45]. The result of this study indicates that the treatment of the test bacteria with the methanolic extract of *R. chinensis* induced possible leakage of the cell envelope. The protein content of supernatant of *E. coli* and *S. aureus* increased significantly ($p<0.05$) with an increase in the time interval when treated with the methanolic extract of *R. chinensis* (Fig. 4 AB & Fig. 4C).

Furthermore, the findings of the scanning electron microscope study suggests the possibility for membrane-damaging property of the methanolic extract of *R. chinensis*. It was observed that after treatment with the methanolic extract of *R. chinensis* mill at concentrations of MIC and at 400 mg/mL there was an alteration in the morphology of cells leading to the leakage of cellular materials (Fig. 5 & Fig. 6) as

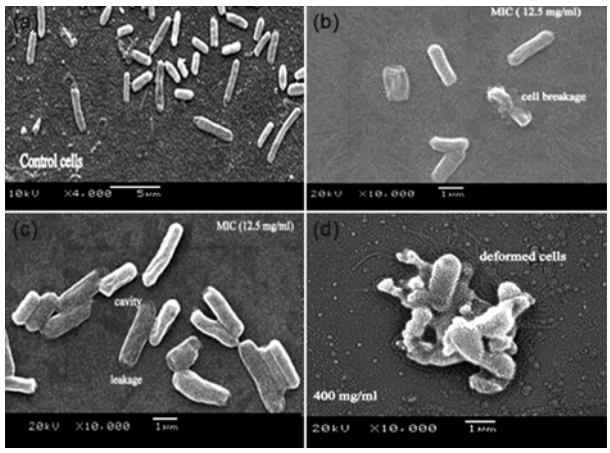

Fig. 5 — (A-D): Scanning Electron Micrographs of (A) Untreated *E. coli* showing normal morphology (B-D) *E. coli* treated with the methanol extract of *R. chinensis* at MIC and at 400 mg/mL respectively showing cellular deformities.

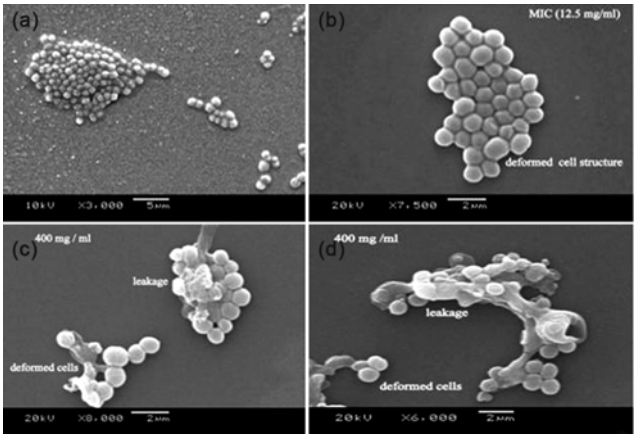

Fig. 6 — (A-D) Scanning Electron Micrographs of (A) Untreated *Staphylococcus aureus* showing normal morphology (B-D) *Staphylococcus aureus* treated with the methanol extract of *R. chinensis* at MIC and at 400 mg/mL respectively showing cellular deformities

compared to the control (untreated) test bacteria. These effects might be due to the various phytochemicals present in the crude plant extract. SEM analysis revealed the possibility of the plant extracts having the potential to target the bacterial cell envelope thereby exhibiting deformed cell morphology.

Conclusion

In this study, the aqueous extract, ethyl acetate solvent extract and methanol solvent extract of *R. chinensis*, showed potent antibacterial property which could be due to the presence of various bioactive compounds extracted in different solvents being used in the study. The bacterial cells treated with the methanolic extract of *R. chinensis* revealed the leakage of cellular materials which exhibited absorbance at the wavelengths of 260 nm and 280 nm. The SEM analysis exhibited adverse cell envelope damaging effects of the methanol extract of *R. chinensis* on the bacterial cells as revealed by deformation in the cell morphology and breakage of cell envelope. Further studies are necessary to elucidate the mode of antibacterial action of the solvent extracts.

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