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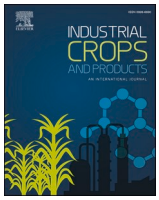

Chemoprofiling of in vitro cell cultures of *Tinospora cordifolia* for protoberberine alkaloid content analysis and to understand their therapeutic potential against *Staphylococcus aureus*

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ABSTRACT

Tinospora cordifolia is an essential part of ethnomedicine, which aids in enhanced immunity and rejuvenates body in combating diseases. Therapeutic potential is attributed to individual or cumulative effects of bioactive metabolites. To provide sustainable source of bioactive metabolites, without affecting plants natural habitat, plant cell cultures are the most suitable alternative source. Current study elucidates the induction and proliferation of in vitro callus cultures from leaf-disc explants of *T. cordifolia* on Murashige and Skoog (MS) medium fortified with 3 μM 6-benzylaminopurine (BAP) and 10 μM 1-naphthalene-acetic-acid (NAA). The cell biomass growth followed the Gompertz kinetic model, predicting maximum fresh and dry weight yields on days 14.46 and 11.71, respectively. Extract was prepared from dried callus biomass using acid-base methodology. Therapeutic protoberberine alkaloids, (jatrorrhizine and palmatine) were identified, purified, and quantified by high-performance liquid chromatography (HPLC). In these callus extracts, 10-fold increase in jatrorrhizine content (435.87 mg g^{-1} DW) and 143-fold increase in palmatine content (567.45 mg g^{-1} DW) were recorded as compared to 40.12 mg g^{-1} DW jatrorrhizine and 3.96 mg g^{-1} DW palmatine present in leaves of field-grown mother plant. Further, confirmation of alkaloids was done using mass spectrometry. These purified alkaloids, jatrorrhizine, and palmatine favored a reduction in the biofilm-forming capability of *Staphylococcus aureus* by 78.32 % and 87.01 %, respectively. Field emission scanning electron microscope (FESEM) analysis of treated sample showed membrane distortion, pore formation, and cytoplasmic leakage in *S. aureus*. The findings aim for sustainable production of protoberberine alkaloids from in vitro plant cell cultures for therapeutic uses.

1. Introduction

Tinospora cordifolia (Willd.) Miers ex Hook. F. & Thomson, is a tropical woody liana of the Menispermaceae family, sharing a long history in serving Ayurvedic and modern medicine, also referred as '*heavenly elixir*'. It functions by providing immunity, longevity and vitality to the body, for fighting against various ailments (Upadhyay et al., 2010). Dwelling in the forests, it has been an essential folklore medicine for tribal and local people of different parts of India and other Asian countries (Upadhyay et al., 2010). The *T. cordifolia* possess diversified utilities and the most utilized part is its stem, followed by its root, leaf and fruit. The stem yield of the plant is noted to be 0.8–1 ton per hectare of Rs 15–20 per kg. Moreover, the requirement for the plant hiked to 1200 tons per year during COVID-19 pandemic 2020–22. The National Medicinal Plants Board (NMPB), Government of India has included *T. cordifolia* in the list of 32 prioritized medicinal plants, conceptualized for germplasm conservation and research (Gowthami et al., 2021).

The bitter flavor and innumerable benefits of the plant are due to the presence of alkaloids. Unlike the phenolic compounds, alkaloids are produced in less quantities in plants. The alkaloidal vitality and their extraction from medicinal plants began in the early 1900s, proclaiming their versatile effects in immunomodulating the human body to fight against diseases (Bisset, 1992). The protoberberine alkaloids, such as palmatine and jatrorrhizine are nitrogen-containing tetracyclic compounds, that belong to the isoquinoline group with tyrosine as their biosynthetic precursor (Grycová et al., 2007). The alkaloids have been found in some other plant species belonging to family, Menispermaceae, Berberidaceae, Ranunculaceae, Papaveraceae, Rutaceae, and Annonaceae (da-Cunha et al., 2005). Although protoberberine alkaloids could be isolated from *Berberis* sp., *Coscinium fenestratum, Coptidis rhizome,* and

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Coptis japonica, the accumulation of alkaloids usually takes 3–4 years (Bisset and Nwaiwu, 1983). *Tinospora cordifolia* tissue culture brings up an excellent alternative for alkaloid production, without its over-exploitation and changes in the metabolite profile. In the present study, in vitro callus cultures were established from leaf-disc explants, with the ultimate aim of isolation of protoberberine alkaloids, particularly, palmatine and jatrorrhizine, from them. This report also highlights the higher productivity of therapeutic alkaloids in plant cell cultures, compared to the leaves of the mother plant.

The targeted therapeutic alkaloids, palmatine and jatrorrhizine are known to have anti-diabetic properties. Liu et al. (2018), displayed chronic *Staphylococcus aureus* infection in the form of biofilm that could lead to insulin resistance, causing impaired glucose intolerance. Thereby, triggering the occurrence and aggravation of diabetes. In the present study, the isolated protoberberine alkaloids were evaluated as potential inhibitors of the biofilm-forming abilities of *S. aureus*. Concerning *T. cordifolia*, several researchers hypothesized the anti-microbial and anti-biofilm effect of crude extracts and berberine (protoberberine alkaloid), against *S. epidermis, Enterococcus faecalis* and *S. aureus* (Bonvicini et al., 2014; Chen et al., 2016; Chu et al., 2016; Jeyachandran et al., 2003). The present study documents the reproducible methodology for production and extraction of protoberberine alkaloids from cell cultures of *T. cordifolia*. The biofilm inhibiting potential of the alkaloids against *S. aureus* was noticeable, posing a remarkable solution of antibiotic resistance by using palmatine and jatrorrhizine as natural antimicrobial agents.

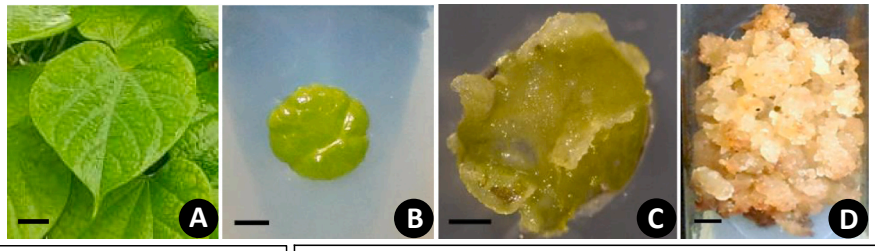
2. Materials and methods

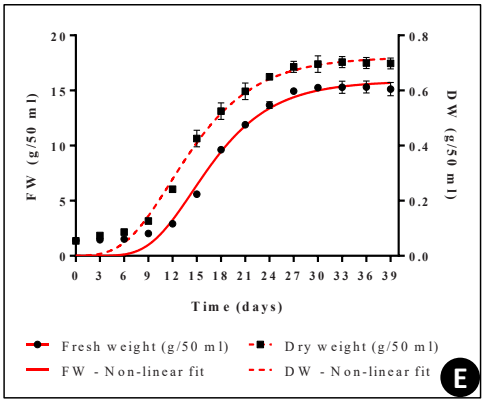
2.1. Plant material and cell culture

The leaf samples (Fig. 1**A**) were collected from the plant growing in the campus of the Indian Institute of Technology Guwahati (Accession Number GUBH 79866, Gauhati University, Guwahati, India), during the months from July to September to raise in vitro callus cultures. The leaves were gently washed with distilled water 4–5 times to remove soil and other superficial contaminants. The explants were then soaked in distilled water for 4–5 h to facilitate the phenolics leaching out. Subsequently, the explants were rinsed with 0.3 % (v/v) Tween-20 for 20 min with gentle agitation, followed by thorough washing with distilled water 4–5 times till the foam disappeared. Under aseptic conditions of laminar-air-flow, the explants were first surface sterilized with a mixture of 0.1 % (w/v) mercuric chloride and 0.3 % (v/v) Tween-20, for 3 min, and finally with 0.1 % (w/v) mercuric chloride for 4 min. The aforementioned steps were accompanied by a thorough wash with sterile distilled water, 4–5 times. Leaf-disc explants of 5 mm diameter were prepared using a cork borer and inoculated on the medium for callus induction. The cultures were observed periodically and the morphological changes were recorded at weekly intervals. A similar medium was used for regular maintenance of the callus lines as well.

2.2. Media optimization for callus induction

Leaf-disc of 5 mm size (Fig. 1**B**) was inoculated on MS basal medium fortified with plant growth regulators, like 2,4-dichlorophenoxyacetic acid (2,4-D), 6-furfurylaminopurine (Kinetin), 6-benzylaminopurine (BAP) and 1-naphthalene acetic acid (NAA), discretely or in various combinations and concentrations. Sucrose (3 %) was added as a carbon source and medium pH was set at 5.8 using 1 N HCl/NaOH before autoclaving. Eighteen leaf-disc explants, one per test tube (Borosil, India; 25 mm dia × 150 mm height), were inoculated per medium composition. Each experiment was repeated thrice. The cultures were incubated under culture room conditions of 1000–1600 lux of light, 25 ± 2 °C temperature, and 50–60 % relative humidity. The inferences were recorded on percent explants showing callus initiation and the time taken for callus induction. Basal MS medium was used as a control.





Gompertz equation:- $y(t) = k \cdot e^{-e^{-r(t-t_0)}}$

Fresh weight	Dry weight
K = 15.86 g/50 ml r = 0.1858 t_0 = 14.46 day R^2 = 0.9806 Adjusted R^2 = 0.9935 Degree of Freedom = 39	K = 0.7212 g/50 ml r = 0.1744 t_0 = 11.71 day R^2 = 0.9840 Adjusted R^2 = 0.9947 Degree of Freedom = 39
$y(t) = 15.86 \cdot e^{-e^{-0.1858(t-14.46)}}$	$y(t) = 0.7212 \cdot e^{-e^{-0.1744(t-11.71)}}$ F

Fig. 1. Establishment of in vitro callus cultures of *T. cordifolia*, using leaf-disc explant, (A) mother plant leaves, **(B)** leaf-disc (5 mm size) inoculated on MS medium supplemented with 3 μM BAP and 10 μM NAA (Bar = 0.4 cm), **(C)** leaf-disc at culture after two weeks, showing callus initiation from the margins of leaf-disc (Bar = 1.8 cm), **(D)** a 90-week-old culture showing proliferated, friable, light-brown callus after 30 passages, each of 21 days (Bar = 0.6 cm), **(E)** fitted curve of biomass growth with time of in vitro grown calluses on MS + 3 μM BAP + 10 μM NAA, using Gompertz kinetic model, **(F)** growth parameters (K = maximum fresh/dry weight (g/50 mL), r = growth rate, t_0 = time of inflection point (where growth rate is max), t = time in days, R^2 = coefficient of determination) and the fitted equation for fresh and dry weight.

2.3. Estimation of dry cell weight

Friable, proliferated calluses, after 3 weeks of inoculation, were harvested for alkaloidal analysis. It was oven-dried at 35 ± 2 ºC for 4 days, till it attained a constant weight. A moderate temperature was maintained to avoid the loss of thermolabile compounds. The dried calluses were stored at − 20 ºC to prevent any kind of degradation. A similar methodology for drying was performed using leaves from the mother plant. The data obtained from fresh and dry weights of in vitro calluses were analyzed using Gompertz kinetic model using GraphPad Prism 6, plotting a relationship between biomass growth with time shown as -

$Y(t) = K \cdot e^{-e^{-r(t-t0)}}$, where, K = maximum biomass in g/50 mL (FW and DW), r = growth rate, t_0 = time of maximum growth rate, and t = time in days.

2.4. Isolation of protoberberine alkaloids

2.4.1. Preparation of crude extracts (method I)

For extraction purposes, 5 g of dried calluses and leaves were taken and pulverized into a fine powder using a porcelain mortar-pestle or mixer grinder. The dried powders were soaked in methanol (Emplura grade, Merck, India) overnight at room temperature under continuous shaking condition. The extract was further sonicated at 35 % amplitude (pulse of 5 s on/off cycle) and filtered using 90 mm Whatman filter paper (Whatman plc, UK). The debris was re-suspended in 10 mL of fresh methanol, re-extracted, and pooled till the color of the re-extract lightened. The pooled filtrate was concentrated to dryness under a vacuum at 50 ºC using a rotary evaporator (IKA, Staufen, Germany). The dry weight of the crude extracts was estimated, before chemical analysis. For HPLC analysis, the samples were re-dissolved in methanol (HPLC grade, Merck, India) and filtered using 0.22 μm of nylon 6,6 membrane (Axiva Sichem Biotech, New Delhi, India).

2.4.2. Preparation of alkaloid-rich fractions (method II)

The crude extracts of in vitro calluses and field-grown leaves were subjected to an additional step in the methodology for extraction of therapeutic alkaloids. The extracts were processed in a hydro-methanolic solvent consisting of 3 % HCl and methanol in an 80:20 ratio; pH ≤ 2.0, with continuous stirring for 2–3 h. The debris was settled on a magnetic surface, while the hydro-methanolic decoction was subjected to fractionation with an equal volume of chloroform. This step was repeated thrice and the chloroform fractions were pooled. The remaining hydro-methanolic decoction was transitioned to basic pH (9–10) with liquid ammonia. Thereafter, the basic hydro-methanolic portion was again fractionated with an equal volume of chloroform thrice. Both the acid- and base-fractionated chloroform fractions were made to paste by evaporating at 50ºC while the basic hydro-methanolic portions were freeze-dried (-100 ºC) using a lyophilizer. The fractions were accurately weighed and re-dissolved in HPLC grade methanol, and filtered via 0.22 μm nylon 6,6 filter for analytical analyses.

2.4.3. HPLC analysis

It was performed using methodology from Kumar et al. (2017) with slight modifications, using the Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a quaternary pump, UV detector, and 100 μl injection loop. The solvents were filtered using a 0.45 μm nylon 6,6 filter and degassed before use. The samples were analyzed at 50 ºC in BDS Hypersil C-18 column (250 × 4.6 × 5 μm; Thermo Fisher Scientific, USA), with an injection volume of 10 μL. The mobile phase comprised A = MilliQ with 20 mM ammonium acetate and 0.03 % formic acid and B = Acetonitrile (20:80) at a flow rate of 0.6 mL min^{-1} and monitored at 345 nm wavelength. Each sample and standard were run in triplicates and the data was integrated and acquired via Lab Solutions software (Shimadzu, Kyoto, Japan). Standards stock solution (1 mg mL^{-1}) of palmatine and jatrorrhizine (Sigma-Aldrich, Japan) was prepared and serially diluted from 500 to 3.12 μg mL^{-1} concentrations for calibration studies. The chromatogram peaks for jatrorrhizine and palmatine were identified by comparing the retention time with their respective standards. The HPLC method validation parameters, such as precision (intra- and inter-day analysis), sensitivity in terms of limit of detection (LOD), and limit of quantification (LOQ) were also estimated. The later was as per International Council for Harmonization (ICH) and Indian Pharmacopoeia (IPC) guidelines.

2.4.4. Mass spectrometric analysis

The purified compounds were re-dissolved in HPLC grade methanol (Spectrochem, India) for mass spectrometry analysis using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF; Bruker-Autoflex Speed, USA) with 355 nm nitrogen laser pulse. α-cyano-4-hydroxycinnamic acid (HCCA) was used as the matrix (blank) for MALDI, and the spectrum was collected in the positive mode and averaged for 100 shots. The MS data was obtained in a scan mode range of 100–1000 amu.

2.5. Biofilm inhibition studies

2.5.1. Microbial strain and culture conditions

S. aureus strain (MTCC 737; procured from NCCS, Pune) was used for the biofilm inhibition studies. It was cultivated in Mueller Hinton (MH) medium by continuous stirring, overnight, at 180 rpm and 37 ºC temperature. Before analysis, the optical density (OD) of the bacterial culture was adjusted to 0.5 (~ 1×10^8 CFU mL^{-1}) by following the McFarland standard method. Then 4 μL (~ 6×10^5 CFUs) of the microbial culture was added to each well containing the purified compounds of jatrorrhizine and palmatine. The experiments were conducted in triplicates and represented as mean ± SD.

2.5.2. MIC determination and biofilm assay

The HPLC-purified alkaloids, jatrorrhizine, and palmatine, dissolved in 2 % DMSO (Merck, India), at serial dilution concentrations of 1000 μg mL^{-1} to 3.12 μg mL^{-1} were used for the study. Kanamycin (20 μg mL^{-1}) was used as a positive control, while 2 % DMSO was used as a negative control. MIC determination was performed using the broth dilution method in 96-well microtiter plate format. The plates were incubated for 24 h and 48 h incubation at 37ºC. The microbial growth was measured via microplate reader (Tecan, Switzerland) at 600 nm. The MIC was determined based on no visible growth (clear wells) after 24 h of treatment.

Each purified alkaloid (3.12–1000 μg mL^{-1}) of 100 μL volume was added to the wells, individually, along with overnight grown microbial culture (~ 6×10^5 CFU mL^{-1}). The mixture was incubated for 24 h and 48 h duration at 37 ºC. Thereafter, the supernatant was discarded and the wells were washed with distilled water thrice to remove the planktonic bacterial cells. Then the remaining surface-associated bacterial biofilm in the well was stained with 0.1 % safranin (Sigma-Aldrich, Japan), for 30 min. Following this, the wells were rinsed with distilled water to remove excess dye. Finally, biofilm-bound safranin was released using 30 % acetic acid (Merck, India) and the absorbance of the supernatant was measured at 530 nm. Each sample was run in triplicates, while the experiments were repeated twice. Minimal biofilm inhibitory concentration (MBIC) after safranin staining was calculated as:

$$\text{Inhibition\%} = (OD_{control} - OD_{sample})/(OD_{control}) \times 100$$

2.5.3. Ultrastructural analysis

The microorganism was co-incubated with purified alkaloids (MBIC concentration) overnight, with a cover-slip as adherent material, by

following biofilm inhibition methodology. Thereafter, the coverslips were washed with distilled water thrice for removal of planktonic cells and kept for fixation in 2.5 % glutaraldehyde (Merck, India) for 5–6 h. The cells were subsequently dehydrated in ethanol (Merck, India) series (10–90 %) for 10–15 min each, gradually, and kept for air-drying. The cover-slip adhered cells were eventually spur-coated with gold before analysis and scanned using FESEM (Zeiss, Germany).

3. Results and discussion

3.1. Initiation of in vitro callus culture of T. cordifolia

The present study utilized MS basal medium supplemented with plant growth regulators, BAP, and NAA, either individually or in various combinations and concentrations to induce callus from leaf-explants (Table S1). Among all the tested media compositions, MS + 3 μM BAP + 10 μM NAA gave 100 % response on callus induction within 15 days (Fig. 1**C**). Callus was initiated from the margins and midrib of the inoculated leaf-disc. The application of growth regulators is essential for dedifferentiation of explants to form calluses since MS basal medium (control) did not support the phenomenon. During initial subcultures, the calluses were hard and dark brown in texture. Regular subcultures of calluses at short intervals of every 21 days favored a decrease in the browning of calli, which later resulted in massively grown, whitish-brown, and friable calluses (Fig. 1**D**). In a report, Chintalwar et al. (2003) documented callus development from inter-nodal segments on MS medium with 1 mg L^{-1} 2,4-D and 0.2 mg L^{-1} kinetin, which were dark brown but friable on semi-solid and liquid medium. Roja et al. (2005) found MS + BAP + 2,4-D for callus induction and berberine detection. While Kumar et al. (2017) utilized BAP and NAA in combination for callus induction and proliferation. In the present study, BAP, when used alone, led to only ≤ 50 % callus initiation. While, NAA was found better for commencement of callusing at higher concentrations (10, 15, and 20 μM). Similarly, Pillai and Siril (2019) reported higher callus induction on 5 μM NAA as compared to IBA, 2,4-D and picloram-fortified MS media.

3.2. Gompertz model-based growth kinetic study

In the present study, the biomass growth curve of in vitro callus followed a sigmoidal pattern, indicating lag phase till 10.5 days, followed by rapid cell division in the exponential phase from 10.5 to 18.5 days and further beginning the stationary phase with asymptotic growth. Maximum fresh weight biomass was 15.86 g/50 mL of medium on 14.46 days (t_o), while it was 0.72 g/50 mL on 11.71 days (t_o) as per the dry weight estimate (Fig. 1**E**). In the Gompertz model analysis, coefficient of determination (R^2) was noted to be 0.9806 and 0.9940 for FW and DW of calluses, respectively, signifying an excellent fit. With this smaller data set, adjusted R^2 showed better accuracy of 0.9935 (FW) and 0.9947 (DW), respectively (Fig. 1**F**). The kinetic modeling approach details biomass growth dynamics, redirects future predictions, and is necessary for large-scale biomass production. The cells were harvested in the early stationary phase (21st day of the subculture), signifying secondary metabolite production. A recent study detailed the utilization of kinetic modeling in understanding growth and metabolite production in adventitious root cultures of *Spilanthes paniculata* (Pachauri and Chaturvedi, 2025). A similar harvested callus was utilized for the extraction of target alkaloids. In this context, Kumar et al. (2017) performed subculture of leaf-induced calluses after 3 weeks, which was further utilized to raise cell suspension cultures. Later, the authors found the highest biomass and protoberberine alkaloids on the 16th day, since cells in a homogenous supply of nutrients shorten the growth cycle. Unlike, Rao et al. (2008) raised leaf-induced in vitro cell cultures of *T. cordifolia* and inferred maximum biomass on the 30th day of cell suspension culture while maximal productivity of berberine was obtained on the 25th day of culture.

3.3. Identification and quantification of protoberberine alkaloids

The compounds were separated based on their polarity difference, with jatrorrhizine being eluted early at the retention time of 6.8 and palmatine at 7.4 min in the respective standards (Fig. 2**A–B**) and in leaf and callus samples (Fig. 2**C–D**). A calibration graph was generated for jatrorrhizine and palmatine as per serially diluted concentrations, aiding in the quantification of both alkaloids in leaf and callus samples. Linearity studies revealed regression coefficient (r^2) value > 99 % while the precision studies inferred CV% to be < 5 % as per the intra-and inter-day analysis (Table 1). The LOD and LOQ for jatrorrhizine were recorded as 2.14 μg mL^{-1} and 6.49 μg mL^{-1}, respectively, whereas, for palmatine, it was recorded as 0.76 μg mL^{-1} and 2.30 μg mL^{-1}, respectively. Additionally, callus extract was spiked with a known amount of alkaloid standards, for the confirmation of compounds indicating jatrorrhizine and palmatine, respectively (Fig. 2**E–F**). Kumar et al. (2017) reported early elution of jatrorrhizine at 11.8 min, followed by palmatine at 13.3 min, in *T. cordifolia*.

The present study reports an increased yield of jatrorrhizine as 435.87 mg g^{-1} DW by 10.8 fold and palmatine as 567.45 mg g^{-1} DW by 143-fold in vitro callus in comparison to the leaf extract (Table 1). The chromatogram showed low elution of polar phenolic compounds, as compared to maceration with methanol. This is because the natural form of alkaloids occurs in complexes with non-alkaloidal molecules (mineral salts, tannins, phenolics, lipids, gums) and their complete removal is quite challenging (Svendsen and Verpoorte, 1983). Generally, alkaloids are hypothesized to be stable in acidic aqueous extract and protoberberine alkaloids are semi-polar, therefore, the neutral form of alkaloid was soluble in chloroform fraction. While the remaining salt/hydrochloride form of alkaloid (after acidification with HCl) was liberated on treatment with ammonia, and was easily solubilized in chloroform. The current extraction (Method II) used an acid-base-organic solvent methodology, which gave 12.8-fold and 149.78-fold improved yield from in vitro callus extracts, as compared with maceration with methanol (Method I).

The present study illustrates dual benefits in providing higher metabolite yield with added profits of better alkaloid extraction, especially from cell cultures than the intact mother plant. Similar studies are available in which plant cell cultures have provided higher productivity of compounds than their wild type. Furuya et al. (1983) noted the higher alkaloidal content, particularly jatrorrhizine, in in vitro calluses (0.3–0.9 % DW) of *Dioscoreophyllum cumminsii* as compared to the intact plants (0.02–0.03 % DW). Berberine was recorded as 10 % (DW) in the suspension cultures of *Coptis japonica* and 0.01 % (DW) in field-grown plants (Matsubara et al., 1989). Similarly, in *Panax ginseng*, ginsenoside (steroid glycosides) was found as 4.5 % (DW) in intact plants while 27 % (DW) was produced by in vitro callus cultures (Bhojwani and Razdan, 1996; Misawa, 1994). Betulinic acid (triterpenoid) was absent in field grown mother plant but was quantified as 2.1% (DW) in leaf derived in vitro callus cultures (Srivastava and Chaturvedi, 2010). There are reports available where the plant growth regulators play crucial role in biosynthetic pathway of isoquinoline alkaloids. Hara et al. (1994) reported cytokinin effect on increased activity of noncoclaurine-O-methyltransferase. The biosynthesis of isoquinoline alkaloids begins with L-tyrosine as a precursor, forming (*S*)-norcoclaurine via noncoclaurine-O-methyltransferase, proceeding to (*S*)-reticuline as an important intermediate compound. The pathway diverts to jatrorrhizine via tetrahydroprotoberberine oxidase, and finally to palmatine through columbamine O-methyltransferase (isoquinoline alkaloid biosynthesis pathway, KEGG database). In another related report, it was clarified that 2,4-D suppressed the expression of norcoclaurine 6-O-methyltransferase and (*S*)-tetrahydroberberine oxidase, a key enzyme controlling benzylisoquinoline (protoberberine) biosynthesis, which was later recovered upon fortification with BAP. The study was conducted for berberine biosynthetic analysis in *Thalictrum minus* cell cultures (Hara et al., 1995). Further, the effects of NAA,

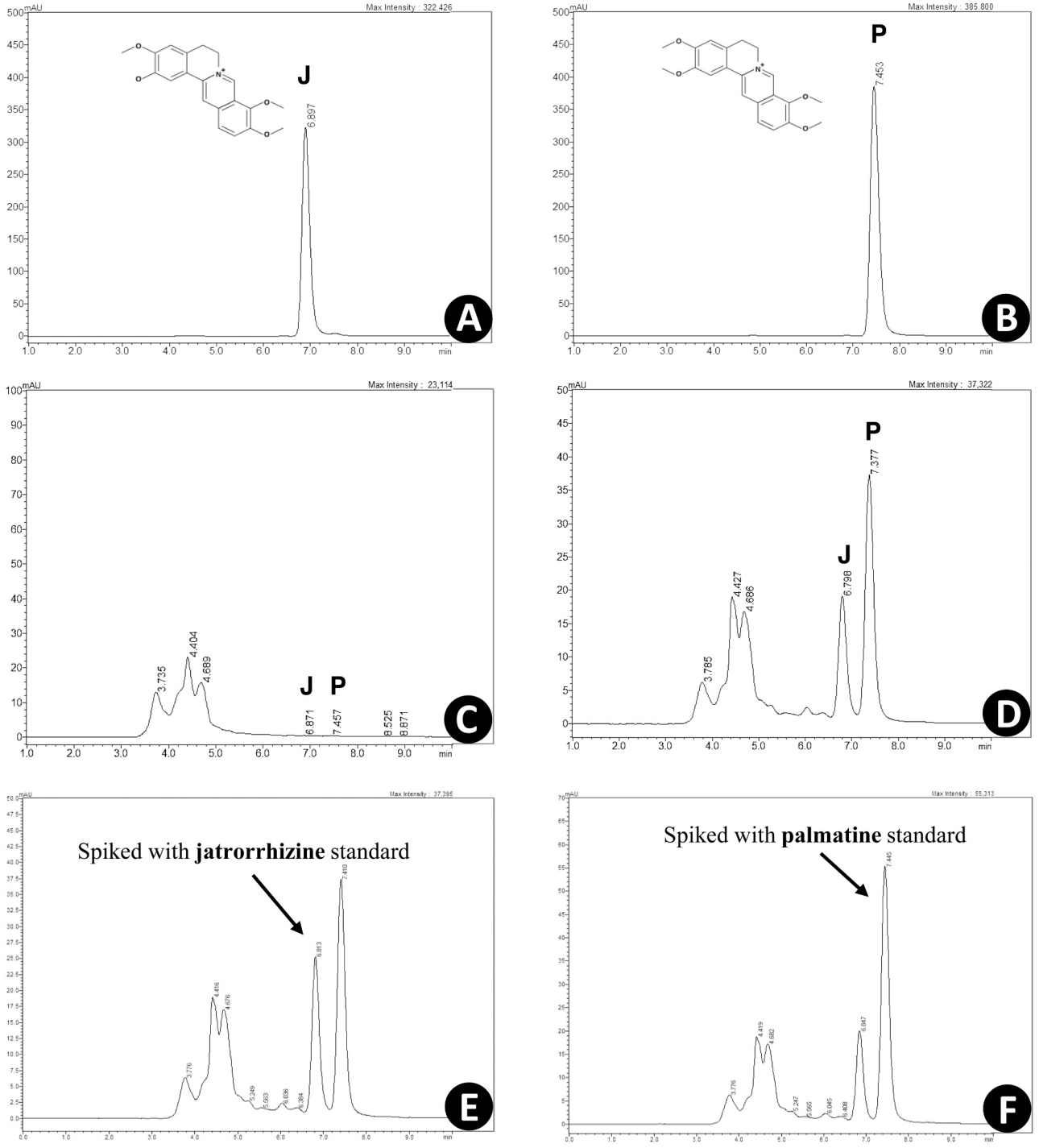


Fig. 2. HPLC chromatograms, (A – B) reference standards of jatrorrhizine, J (RT: 6.8 min) and palmatine, P (RT: 7.4 min), (**C**) field-grown leaf extract of *T. cordifolia*, processed via extraction method II (**D**) in vitro callus extract of *T. cordifolia*, processed via extraction method II, (**E**) extract from (D) spiked with known amount of jatrorrhizine standard, (**F**) extract from (D) spiked with known amount of palmatine standard.

Table 1
Linearity, sensitivity, and precision parameters for quantification of jatrorrhizine and palmatine.

Protoberberine alkaloid	Retention time (min)	Calibration curve[a]			Sensitivity (μg mL^{1})		Coefficient of variation (CV%)		Amount (mg g^{-1} DW)	
		Y-intercept	Slope	r2	LOD	LOQ	Intra-day	Inter-day	Leaf	Callus
Jatrorrhizine	6.8	46,983	72,439	0.9947	2.14	6.49	0.27	1.77	40.12	435.87
Palmatine	7.4	18,414	79,918	0.9978	0.76	2.30	0.11	1.28	3.96	567.44

[a] Values represented as mean ± standard deviation ($n = 3$).

BAP, and light exposure stimulated higher production of berberine in *Thalictrum minus* and *Coscinium fenestratum* cell cultures (Nair et al., 1992; Hara et al., 1993). Gügler et al. (1988) found that yeast glucan elicitor stimulated tyrosine decarboxylase in suspension cultures of *Thalictrum rugosum* and was found to be positively stimulating berberine biosynthesis. Min et al. (2023) mentioned a total of 36 differentially expressed genes and WRKY transcription factor when elicitation targets involved in the isoquinoline alkaloid biosynthesis pathway.

In *T. cordifolia*, berberine content was reported to be 5–14-fold higher than intact plants and the authors suggested growth regulators, such as BAP, NAA, 2,4-D, and medium pH, carbon-source and cell-aggregate size, are responsible this higher berberine production in in vitro cell lines (Rao et al., 2008). Pillai and Siril (2019) successfully retrieved 0.76 mg g^{-1} DW of berberine from stem-induced in vitro callus and it was further enhanced to 1.89 mg g^{-1} DW after 40 μM tyrosine (precursor) treatment to these callus cultures of *T. cordifolia*. *Piriformospora indica*, a potent fungal biotic elicitor in the form of filter-sterilized culture filtrate (5 %) improved jatrorrhizine and palmatine content by 4.2-fold (10.72 mg g^{-1} DW) and 4.0-fold (4.39 mg g^{-1} DW) respectively, whereas, methyl jasmonate (250 μM) elicited jatrorrhizine by 2.9-fold (7.34 mg g^{-1} DW) and palmatine content by 4.1-fold (4.43 mg g^{-1} DW) in cell suspension cultures of *T. cordifolia* (Kumar et al., 2017).

3.4. Characterization of purified alkaloids via mass spectrometric analysis

Protoberberine alkaloids were validated using MALDI-TOF, in which, the respective standards represented the mass values of 338.10 *m/z* and 352.14 *m/z* corresponding to jatrorrhizine and palmatine, respectively as shown in Fig. 3**A**. Similarly, HPLC peak eluents of both the compounds from in vitro calli extract, also showed corresponding mass values, such as 338.11 *m/z* signifying the presence of jatrorrhizine with the fragmentation of 324.09 *m/z* ($[M]^+$-CH_3 + H^+) (Fig. 3**B**). While palmatine was denoted as 352.14 *m/z* with the fragmentation of 338.11 *m/z* value ($[M]^+$-CH_3 + H^+) (Fig. 3**C**). Analyzing the samples in the positive mode led to the addition of protons to the fragments as well. In a similar view, the mass spectrometry of purified jatrorrhizine and palmatine in in vitro cell cultures of *T. cordifolia* was performed through ESI-QTOF-MS depicting their respective monoisotopic masses (Kumar et al., 2017). Moreover, Li et al. (2015) conducted a detailed MS^n study for the fragmentation behavior of important protoberberine alkaloids through UHPLC-orbitrap mass spectrometer. The changes in the structure were elucidated with the probable biosynthetic pathway of protoberberine alkaloids in zebrafish model.

3.5. Biofilm inhibition studies of S. aureus

3.5.1. MIC determination and biofilm assay

Staphylococcus aureus is a chronic infection-causing microbe affecting the upper respiratory tract and the biofilm formed highly affects the medical implants and wounds (Kluytmans et al., 1997). The susceptibility of jatrorrhizine and palmatine was examined in the growth reduction of planktonic cells of *S. aureus*. The anti-bacterial assessment was carried out for 24 h and 48 h duration to analyze the chances of re-occurrence especially in the case of biofilm-forming bacteria. MIC of both the purified alkaloids displaying no visible growth was recorded to be 250 μg mL^{-1} after 48 h of treatment (Fig. 4**A–B**). Lower concentrations (< 50 μg mL^{-1}) were found ineffective in providing anti-bacterial efficacy. The present study has also monitored the optimum range of concentrations that should not generate resistant, non-treatable organisms (Wiegand et al., 2008). Kanamycin was used as positive control which binds to 30S ribosomal subunit and inhibits protein synthesis. It also causes fissures in the bacterial cell membrane.

Consequently, the biofilm-forming capability of *S. aureus* was tested at similar concentrations. Biofilm is considered to be a mature and huge bacterial community, adhered to surfaces and supported by exopolysaccharides, proteins, and nucleic acids and interconnecting via

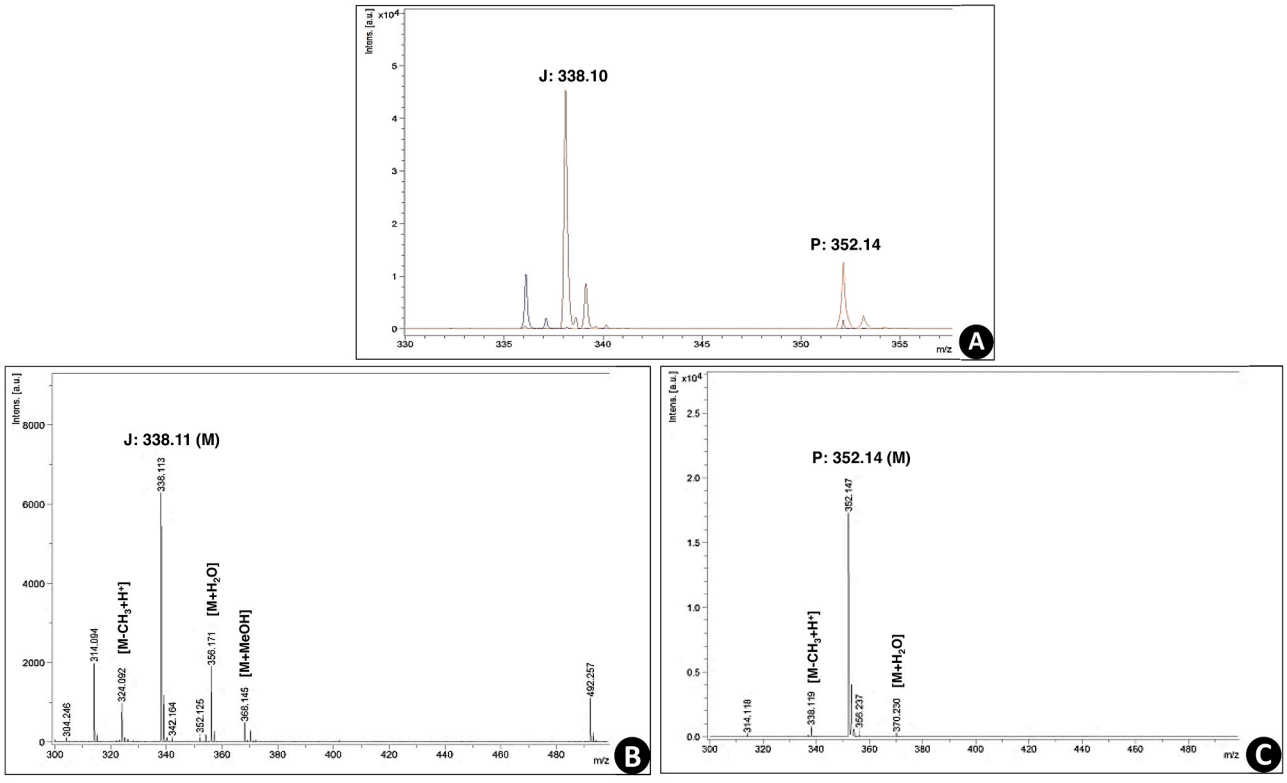


Fig. 3. Mass spectrometric confirmation of jatrorrhizine (J) and palmatine (P) at 338.10 *m/z* and 352.14 *m/z* values, in, (A) standard mix of the alkaloids, (B) and (C) callus extract purified from Method II. M represents molecule.

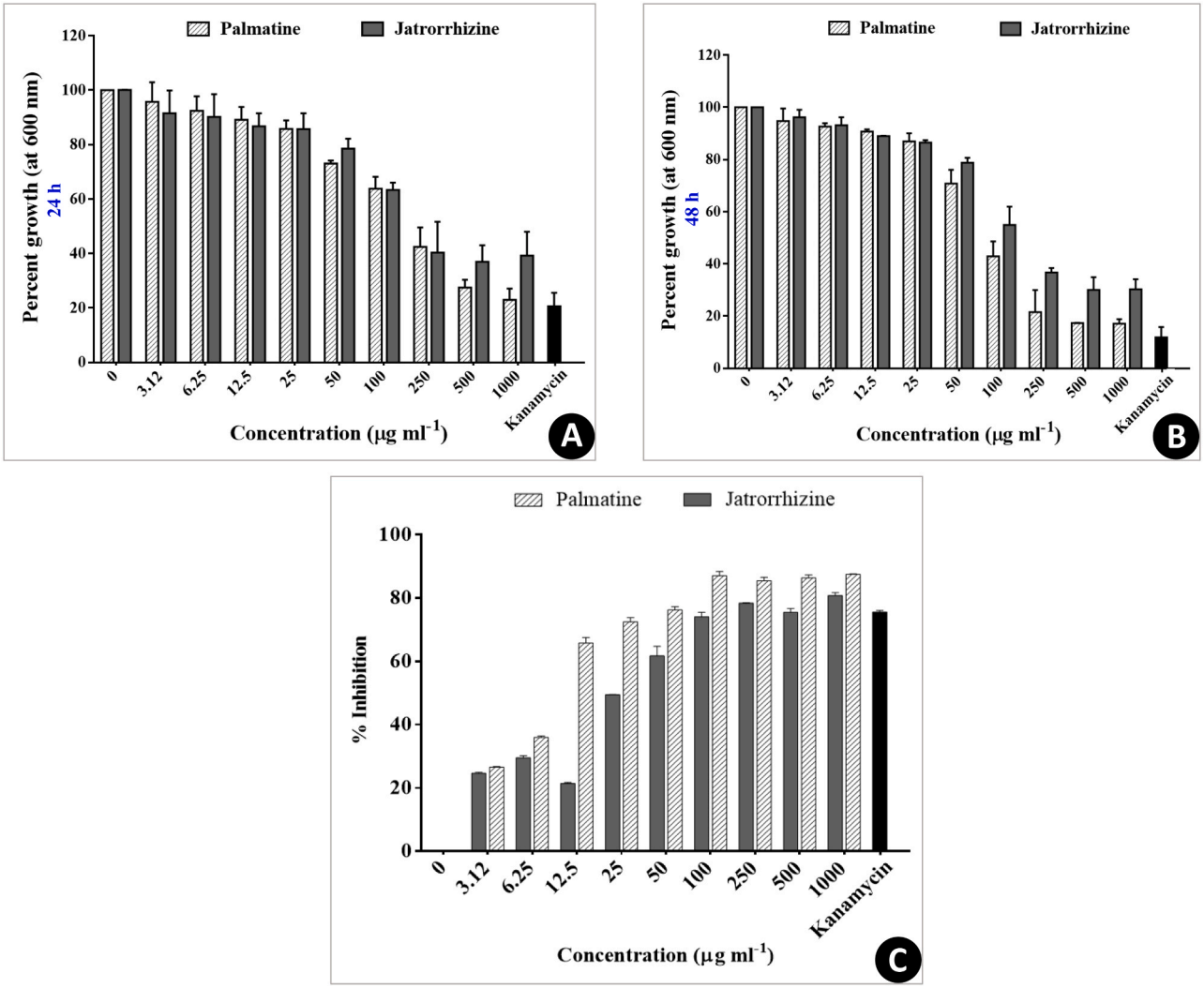


Fig. 4. Quantitative analysis of antimicrobial potency of palmatine and jatrorrhizine, where, (A, B) MIC determination of the compounds at 24 h and 48 h of treatment, respectively, upon two-way ANOVA (Bonferroni test), interaction factor between control and treatment (3.12–25 μg mL^{-1} is non-significant ($p > 0.9999$), **(C)** biofilm inhibition assay after 48 h incubation with purified alkaloids, palmatine and jatrorrhizine. All values are represented as mean ± SD ($n = 3$) of three-independent experiments. Statistical analysis using two-way ANOVA indicated all interactions between control and treatment as significant with $p < 0.0001$.

signaling molecules (Mohammed et al., 2018). Safranin dye which effectively binds to the glycosaminoglycan, component of ECM, was used for analyzing the biofilm-forming capability of the bacteria (Kiviranta et al., 1985). As per Fig. 4**C**, the inhibition% of *S. aureus* was calculated displaying an 87.01 % reduction in the case of palmatine at MBIC of 100 μg mL^{-1} upon 48 h' treatment, while jatrorrhizine (MBIC at 250 μg mL^{-1}) exhibited 78.32 % reduction. The compounds decreased the formation of biofilm in a dose-dependent manner. The study was effectively visualized by the safranin coloration which was directly proportional to the biofilm formation. Apart from safranin, crystal violet can also be used as an alternative for quantitative estimation of biofilm (Corte et al., 2019). Among the two alkaloids, palmatine was found to be more effective for biofilm inhibition than jatrorrhizine. Similar results were observed through qualitative microscopic analysis using fluorescence microscopy (*data not shown*). In this context, another isoquinoline alkaloid, berberine was found to be quite effective against antibiotic resistance pathogens. Xia et al. (2022) reported the cell wall and cell membrane disintegration of *S. aureus* upon berberine treatment. Berberine was found to interfere with the nucleic acid, cell wall, cell membrane transport, and metabolic and motility functions of *E. coli* (Karaosmanoglu et al., 2014). In another study, 80 μg/mL of palmatine treatment for 8 h stimulated transepithelial migration and inhibited mucosal inflammation (Hui et al., 2020). On the other hand, jatrorrhizine was found potent against *Brucella abortus* than other protoberberine alkaloids, considering the presence of free hydroxyl group at C-2 and C-3 positions (Azimi et al., 2018).

3.5.2. *Ultrastructural evaluation of bacterial cells upon alkaloidal treatment*

Upon treatment at their respective MBIC concentration (jatrorrhizine: 250 μg mL^{-1} and palmatine: 100 μg mL^{-1}), abnormal structural changes, such as membrane deformation or damage, pore formations, leakage of cytoplasmic content were observed on discrete or aggregated bacterial cells, as compared with the untreated bacterial cells, which showed smooth and uninjured surface (Fig. 5). The alkaloids were found to be effective in showing the unusual or distressed morphology in the aggregated stage of *S. aureus*. Previous inhibitory analyses were well-correlated and supported by the fact that membrane disruption is one of the inhibitory actions of target alkaloids. Similar observations have also been described in berberine-treated methicillin-resistant *S. aureus* (MRSA) (Chu et al., 2016), *E. coli* (Saikia and Chaudhary, 2018), *S. epidermis* (Wang et al., 2009), *Bacillus subtilis* (Prince et al., 2016).

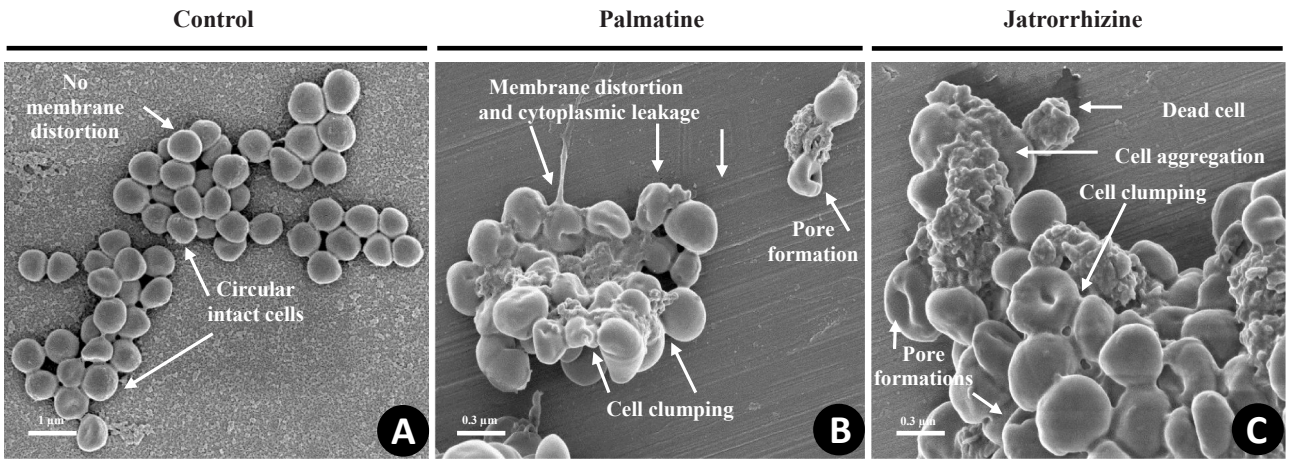


Fig. 5. Ultrastructural morphological analysis of treated and untreated *S. aureus*, (A) bacterial cells without any treatment (control) showing circular intact cells and no membrane damage (Bar = 1 µm), **(B)** palmatine treated bacterial cells depicting membrane distortion, pore formation, and cytoplasmic leakage and (Bar = 0.3 and 0.2 µm), **(C)** jatrorrhizine treated bacterial cells showing the cell aggregation and clumping, altered surface morphology and pore formations (Bar = 0.3 µm).

4. Conclusion

The present study was consolidated on enhancing the yield proficiency of therapeutic protoberberine alkaloids, jatrorrhizine, and palmatine, in in vitro generated callus cultures of *T. cordifolia.* Interestingly, these alkaloids were present in lower quantities in the leaves of field-grown source mother plant of *T. cordifolia* and in other plant species of the Menispermaceae family. In the current study, the step-wise analysis approach aided in optimizing the separation and isolation profiles of the alkaloids, a necessary tool for industrial scale production. The alkaloids were found to be highly persuasive in reducing the biofilm formation by *S. aureus*, as confirmed in quantitative and ultrastructural studies. This work provides crucial information on optimized parameters for production of palmatine and jatrorrhizine-based therapeutic drugs.

CRediT authorship contribution statement

Rakhi Chaturvedi: Writing – review & editing, Validation, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Vartika Srivastava:** Writing – original draft, Visualization, Investigation, Formal analysis, Data curation.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Rakhi Chaturvedi reports a relationship with Indian Institute of Technology Guwahati that includes: employment. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Conflict of interest

The authors declare no conflict of personal or financial interest to influence the work reported in this article.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2025.121573.

Data availability

Data will be made available on request.

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