Original Article

MICROBICIDAL POTENTIALITY OF PURIFIED ANTHOCYANIN FROM *IN VITRO* CULTURE OF *CLERODENDRON INFORTUNATUM* L. AGAINST SELECTED PATHOGENS

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ABSTRACT

Objective: *Clerodendron infortunatum* L. is a widely used medicinal herb over centuries for curing many skin-borne disorders. The present study was designed to validate the tribal knowledge by evaluating antimicrobial potential of purified anthocyanin extracted from *in vitro* cell suspension culture.

Methods: The explants were inoculated on murashige and skoog (MS) medium mixed with various combinations of 2, 4-D a+BAP for callus induction. Green compact callus was initiated within 30 d from the explants on MS medium fortified with benzylaminopurine (BAP) (2.0 mg/l)+2, 4-D (0.5 mg/l). Subsequently, anthocyanin was triggered from the compact callus by subculturing in the medium containing 2, 4-D and Kinetin. Cell suspension culture was also developed. Anthocyanin production was enhanced by elicitation using salicylic acid and others. Three chromatographic methods such as solid phase extraction by Sepharose C18 column, Oasis-MCX and Amberlite XAD 7+Sephadex LH 120 sorbents were used to purify the *in vitro* synthesized anthocyanin from the cell cultures. HPLC and molar absorptivity assay were carried to check the purity. Antimicrobial analysis was also carried using standard protocols to check minimum inhibitory concentration (MIC) and minimum killing concentration (MKC).

Results: The mean purity values obtained by high-performance thin layer chromatography (HPLC) were 90.9%±1.9, 80.60%±2.3 for Oasis MCX, Amberlite XAD-7+Sephadex LH-20 column respectively. However, the purity by molar absorptivity was found to be less. HPLC chromatogram revealed 12 fractions of anthocyanin. Inhibition zone diameter, MIC and MKC values obtained for the purified anthocyanin revealed its antimicrobial potentiality but at different levels among the selected bacteria and fungi. *C. albicans, S. aureus*, *P. aerugenosa* showed significant values followed by MRSA, *E. coli and A. flavus*. The results are comparable with the synthetic antibiotics. However, *E. faecalis* was more resistance. Mode of action was confirmed from the results of intracellular potassium leakage and bacterial membrane integrity analysis.

Conclusion: Thus, the study confirms the efficacy of anthocyanin as natural antimicrobial and suggests the possibility of employing it as drugs for the treatment of infectious diseases caused by the pathogens.

Keywords: *Clerodendron,* HPLC, MS medium, Hormones, Anthocyanin, Column chromatography, Antimicrobial

INTRODUCTION

Antibiotics are one of the most important drugs in combating pathogenic infections and to safeguard the health-related quality of human life. However, over the past many decades, these antibiotics have become less effective against many pathogens and also produce toxic reactions. Similarly, the emergence of drug-resistant bacteria coupled with resistance against the common synthetic antibiotics created health hazards among human beings. Therefore, it is essential to find out new plant-based drugs with more potentiality. Drugs derived from herbal sources play a significant role in the prevention and treatment of many common diseases. In the least developed countries, ethnic medicine becomes part of their primary healthcare systems [1]. Herbals are commonly exploited in the ethnic medicine and their curative properties are well documented. Nearly, about 61% of herbal drugs are developed based on phytochemicals and they have been effective especially against infectious disease and lifestyle diseases [2]. However, recently the rate of active novel chemical entities related to microbicidal is declining [3]. Secondary metabolites of flowering plants may give a new source of microbicidal potential with possible novel mechanisms of action [4]. The bactericidal effects of ethno medicinal plant extracts have been studied by a large number of researchers around the world.

Plants possess a pool of secondary metabolites like tannins, terpenoids, alkaloids, flavonoids, glycosides, etc., which have been proved as antimicrobial under *in vitro* conditions. From pre-historic periods, herbal medicines have been used by common man. Medicinal potentiality of different indigenous herbals for many disorders has been documented by practitioners of traditional medicine [5]. Microbicidal powers of medicinal herbals are being increasingly reported from different parts of the earth. WHO estimates that herbal extracts or their active molecules are used as folk medicine in traditional therapies i.e., approximately 80% of the world population. Antibiotic resistance by microorganisms has increased due to their genetic ability to transmit and acquire resistance to synthetic drugs which are utilized as therapeutic agents.

In this juncture, Clerodendron infortunatum L. of Verbenaceae has been selected. In Ayurvedic literature, the species has been described to be useful against skin disorders and its use into the treatment of hematemesis, and leucoderma has been suggested [6]. Further, their pharmacological uses as pest and disease control agents were reported. Similarly, this plant is widely used by tribal people to treat various ailments including ringworm and other fungal skin infections. No scientific data was available regarding anthocyanin, in vitro culture and its microbicidal potentiality of C. infortunatum. In this scenario, the present study was aimed to validate the ethnic knowledge of its microbicidal potential using anthocyanin.

MATERIALS AND METHODS

Plant material

Clerodendron infortunatum was collected from wild habitats of Kallar region, Ponmudi hills of Western Ghats. Identity was carried by referring floras and confirmed by referring herbarium of Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode and voucher specimen was deposited in the Department of Botany, herbarium (UCB 2369).

Plant material and *in vitro* culture

Fresh excised leaves and nodes of *C. infortunatum* were employed as explants for culturing in the MS medium culturing [7]. The explants were surface sterilized, disinfected with teepol (5% v/v) for 20 min

followed by mercuric chloride treatments (0.01-0.2%, for 1-5 min). Subsequently, the explants were thoroughly washed with deionized water for thrice. The sterilized explants were dissected out into a suitable size and inoculated on MS medium fortified by sucrose (3%) in 0.6% agar for solidification. pH was maintained to 5.8, prior to autoclaving at 15 lb pressure or 121 °C for 20 min. The cultures were kept at 25 °C with a photoperiod of 12 h.

The hormonal combinations in the MS medium supplemented were 2,4-dichlorophenoxyacetic acid (2,4-D)+Benzyl amino purine (BAP) (0.5-3.0 mg/l) and BAP+NAA (0.5-3.0 mg/l) for induction of callus. Callus obtained were sub-cultured in fresh MS medium with 2,4-D+kinetin (0.5-3.0 mg/l) at different concentrations to generate pigmented callus.

Cell suspension cultures were derived from friable callus in 250 ml Erlenmeyer flasks containing 100 ml of liquid MS medium fortified with various concentrations of 2,4-D+Kin (0.5 to 3 mg/l) in triplicates. The cell suspensions were kept at 110 rpm on a rotary shaker at 25±1 °C, 3000 lux and 16/8 h photoperiod. Cultures were retained for one month and their growth was determined by loss of weight by dissimilation.

Analysis was continued from 10th to 25th days using different types of elicitors such as salicylic acid, ethephon and precursors like phenylalanine and shikimic acid into the suspension cultures. Chemicals were sterilized through 0.22 μm milipore filters and added at the time of inoculation to make the concentration of 50 μl/25 ml suspension culture. The cultures were maintained at 25±1 °C under the continuous white fluorescent light on a rotary shaker.

Estimation of anthocyanin content

1 g of *in vitro* pigmented cell mass was used for isolation and estimation of anthocyanin. The OD was recorded at 510 and 700 nm against distilled water as blank [8].

Purification

Crude anthocyanin extract was purified using three different protocols and was fractionated by HPLC-PDA.

(a) Sepharose packs solid-phase extraction (SPE. C18) was used. Giusti *et al.*, [9] method were employed to yield maximum anthocyanin fraction. (b) Oasis-MCX SPE is a mixed mode between cation exchange and reversed-phase interactions. (c) Amberlite XAD-7 and Sephadex LH-20 SPE i.e., purification through 2 different cartridges. Initial purification was done with Amberlite XAD 7, and the resulted fraction was loaded into Sephadex LH 20 cartridge to separate anthocyanins from proanthocyanidins.

(b) HPLC-PDA and MS analysis were carried using Shimadzu HPLC-photodiode array (PDA) system equipped with a SPD-M20A PDA detector and also Shimadzu LCMS-2010 EV liquid chromatography. The mobile phase was solvent A: 4.5% formic acid in LC/MS grade water, and B: 0.1% formic acid in LC/MS grade acetonitrile.

Microbial cultures and growth condition

Freeze dried cultures of *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Candida albicans* ATCC 66027, *Aspergillus flavus* ATCC 16888 were purchased from Institute of microbial technology (IMTECH), Chandigarh. All test strains were re-isolated three successive times on Mueller Hinton agar, MHA (Oxoid) to purify and identity was confirmed by standard bacteriological methods [10]. The inoculum size of each test strain was standardized according to the Committee for Clinical Laboratory Standards [11]. The test bacterial strain was inoculated into Mueller Hinton broth, MHB (Oxoid) medium and incubated for 3–6 h at 35 °C in a shaker water bath until the culture attained a turbidity of 0.5 McFarland unit. The final inoculum was adjusted to 5×10^5 cfu/ml.

Tube dilution assay

The MIC values of purified anthocyanin from *C. infortunatum* were determined using two-fold broth microdilution to prepare extract concentrations of 100, 50, 25, 12.5, 6.3, 3.2, 1.6, 0.8, 0.4, and 0.2 mg/ml; 1 ml of anthocyanin was added to test tubes containing 1 ml of sterile MH media. The tubes were then inoculated with a drop of microbial suspension and incubated at 37 °C for 24 h. Amphotericin B and tetracycline (0.05 mg/ml) were used as positive controls for the fungus and bacteria, respectively. Water was used as the negative control. The MIC value was determined macroscopically after 24 h of incubation in comparison with the growth and sterility controls [10, 11, 12]. MH plates were grouped into six different classes and labeled with the diverse concentrations on the base of the plates; these were used to plate out the contents of each tube in the respective sections of the plates. The plates were incubated for 18-24 h at 37 °C, after which the MBC were recorded. Six replicates were done for each extract concentration and controls against the bacteria and three replicates for the fungi [11].

Well diffusion assay

Antimicrobial susceptibility testing was done using the well diffusion method to detect the presence of anti-bacterial or anti-fungal activities of the plant samples [13]. A sterile swab was used to evenly distribute bacterial or fungal culture over the appropriate medium as stated previously. The plates were allowed to dry for 15 min before use in the test. Wells were then created and a pipette was used to place 30 μl of anthocyanin from *C. infortunatum* into each well. The same extract was used on each plate; with a total of two plates used for each extract including two wells for the positive and negative controls. The negative and positive controls were the same as used in the tube dilution assay. The plates were incubated at 37 °C for 24 h after which they were examined for inhibition zones. A caliper was used to measure the inhibition zones. Twelve replicates were done for each of the different concentrations, and each experiment was repeated six times to ensure reliability.

Potassium (K+) leakage

The potassium leakage was determined using a flame emission and atomic absorption spectroscopy used for titration in solution following the protocol of Edris *et al.*,[14]. The solution was filtrated after contact with the test compounds. The samples were analyzed in a GBC AAS 932 plus device using GBC Avante 1.33 software.

Membrane integrity analysis using propidium iodide uptake

The Live/Dead BacLight kit (Invitrogen) assesses membrane integrity by selective stain exclusion method of Simoes *et al.*, [15]. This is a rapid method commonly used to determine both viable and total counts of bacteria [16].

Statistical analysis

The results of the analysis were expressed as the means of three independent analyses. The results of antimicrobial activity was analyzed by one-way analysis of variance (ANOVA) followed by Tukey test for multiple comparisons. The level of significance was set at 95%. Statistical analysis was performed with statistical software SPSS 17 (SPSS for Windows; SPSS Inc, Chicago, IL).

RESULTS AND DISCUSSION

Pre-treatment with 0.1% $HgCl_2$ (2 min) was optimal to establish explants with a reduced rate of contamination and also showed maximum establishment (95%) on medium. The leaf and nodal explants inoculated on MS medium fortified with various combinations of 2,4-D and BAP for callus induction. After 30 d, leaf explants were proliferated into green compact callus (fig. 1) remarkably. Callogenesis revealed that the fresh weight of all the calli increased in MS media combinations of BAP+2,4 D (0.5–3 mg/l) compared to BAP+NAA (0.5–2 mg/l). The MS medium supplemented with 2 mg/l BAP+0.5 mg/l 2,4 D yielded the highest (2. 7±0.45 g) callus fresh weight compared with other treatments. All the media combinations when fortified with BAP significantly improved the callus fresh weight indicating its importance for callus growth in *Clerodendron* comparing to 2, 4-D and NAA. The resulted callus was further subjected to sub-culturing to induce anthocyanin synthesis.

Further, sub-culturing of callus was attempted on MS medium fortified with 2,4-D and kinetin at different concentrations. Luxuriant pigmented callus was achieved on MS medium supplemented with 1 mg/l 2,4-D+2 mg/l kinetin after 60 d of subculture i.e., 2.68±0.66%. Increase in the concentration of 2,4-D

and kinetin up to 5 mg/l decreased callogenic potentiality of the explants and which in turn the pigmentation (fig. 2). Interestingly, light is a major factor influencing the green callus formation with protocorm-like proliferation at 25 °C and 12 h photoperiod with an irradiance of 60 ± 2 µmol/m²/sec.

Fig. 1: Green compact callus on MS medium supplemented with 2.0 mg/l BAP+0.5 mg/l 2,4-D (30 d) of *C. infortunatum* L

Fig. 2: Production of pigment callus of *C. infortunatum* in MS medium fortified with 2,4-D (1 mg/l) and kinetin (2 mg/l) (Kn)

Batch suspension cultures in triplicate were initiated with 2-3 g of friable callus as an inoculum at temperature 25 ± 1 °C having photoperiod of 16-8 at 110 rpm in liquid MS medium supplemented with the same combinations of the growth regulators as those used in callus culture yielded transparent, homogeneous and non-chlorophyllous cultures which were used to evaluate the growth of biomass and anthocyanin content. Growth pattern of each culture was determined by loss of weight dissimilation method. Interestingly, the cultures yielded sigmoid curves with five growth phases i.e. lag, exponential, linear, stationary and progressive decline. The growth rates of cells were initially slow in the lag phase but as the cultures proceeded, the growth increased significantly and accumulated a great

amount of fresh weight (4 fold) over a period of 20-25 d then the growth of cells became stable and started declining from 30th day. Maximum growth was achieved in suspension culture supplemented with Kin (2.5 mg/l)+2, 4-D (0.5 mg/l) i.e., 3.86 ± 0.01

Fresh Cell Weight (FCW) showed an increase marginally with salicylic acid (SA) and declined. The maximum fresh cell weight noticed was at 20th day and subsequently declined i.e., 9.8 ± 0.32 FCW. Meanwhile, the anthocyanin content increased and attained a maximum value of 4.5 ± 0.22. Similarly, no sound variations were noticed with ethephon on the fresh cell weight and anthocyanin content. Further, phenylalanine and shikimic acid did not showed remarkable values on the fresh cell weight and anthocyanin content.

Quantification of anthocyanin

The anthocyanin content was quantified from the *in vitro* elicited cells and pigmented calli which showed a range from 1.7 to 4.5 mg/g monomeric anthocyanin i.e., approximately 5 fold increase compared with the *in vivo* seeds (0.89 mg/g).

The embryonic nature of agitated cells was analyzed using acetocarmine staining method. Pinkish coloured cells were noticed to indicate active embryonic cells. Subsequently, the anthocyanin extracted from the callus was subjected to TLC. 3 prominent bands were obtained in the chromatogram with yellow, dark violet and green-yellow colours having Rf values of 0.77, 0.65 and 0.511 respectively. Based on the Rf values anthocyanin was predicted as Cyanidin, delphinidin and malvidin based compounds. Further, the crude anthocyanin extract was subjected to purification with 3 different columns and further fractionated by HPLC-PDA analysis.

Purity evaluation by HPLC-PDA and molar absorptivity

The highest purity was found to be with Oasis MCX ($90.9\%\pm1.9$) with molar absorptivity $90.9\pm1.9\%$.

Identification and quantification of anthocyanins

The HPLC chromatogram of purified fractionated anthocyanin comprises 12 peaks (fig. 3). Based on literature data the elution order reported of anthocyanin derivatives were galactosides, glucosides and arabinosides. The fragmentation of delphinidin derivatives in MS2 yielded the formation of delphinidin aglycone *(m/z = 302 [M+H]+)*, after the characteristic release a 160 indicating the galactoside or glucoside in case of compounds 1 and 2, respectively and the release of 131 indicating the arabinoside unit in case of compound 4. Using a similar procedure, the identification of all other compounds was carried. Peaks were identified as 1-Delphinidin-3-O-galactoside, 2-Delphinidin-3-O-glucoside, 3-Cyanidin-3-O-galactoside, 4-Delphinidin-3-O-arabinoside, 5-Cyanidin-3-O-glucoside, 6-Petunidin-3-O-galactoside, 7-Cyanidin-3-O-arabinoside, 8-Paeonidin-3-O-galactoside, 9-Petunidin-3-O-arabinoside, 10-Malvidin-3-O-galactoside, 11-Malvidin-3-O-glucoside, 12-Malvidin-3-O-arabinoside.
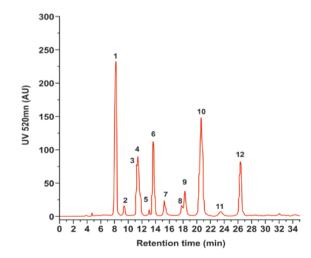

Fig. 3: HPLC-PDA chromatogram of purified anthocyanin of *C. infortunatum* L.

Antimicrobial potentiality

The results illustrated in tables 1 and 2, indicated that purified anthocyanin of *C. infortunatum* showed antibacterial activity against both the Gram-positive (*Staphylococcus aureus*, MRSA, and *E. faecalis*), Gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa*,) and antifungal potential among the selected fungi like *Aspergillus flavus* and *Candida albicans*. However, MKC of the purified anthocyanin extract against the Gram-positive bacteria was ranged from 0.8 to 6.4 mg/ml, meanwhile, among the Gram-negative bacteria the values were 1.6 to 3.2 mg/ml). Similarly, in the case of fungi, the MKC was also between 0.8–3.2 mg/ml) (table 1).

In this study, the growth of MRSA was remarkably inhibited by the purified anthocyanin extract giving an MIC of 1.6 mg/ml by the tube dilution method and the inhibition zone as high as 20.8 mm by the well diffusion method. The anthocyanin extracts an average zone of inhibition for MRSA was less than that of non-methicillin resistant *S.*

aureus (26.5±0.45 mm). The growth of *P. aeruginosa* was also inhibited but to a lesser extent with MIC of 0.8 mg/ml and inhibition zones as high as 22.5 mm (table 2). This is in comparison to the control Gentamicin (one of the commonly used anti-pseudomonal drugs), which produced inhibition zones of 28.6±0.44 mm.

The results of the well diffusion assay corroborates with the results of the tube diffusion assay. For the Gram-positive bacteria, (*Staphylococcus aureus*, MRSA, and *E. faecalis*) zones of inhibition were all above 10.86±0.4 mm for the extract indicating their optimal antibacterial potency. For the Gram-positive bacteria *Escherichia coli* the anthocyanin extract produced the antibacterial activity with optimal MIC and MKC values (1.6 and 3.2 mg/ml) and larger zones of inhibition (16.8 mm) while for *Pseudomonas aeruginosa* the anthocyanin extract was more susceptible (Tables 1 and 2). Purified anthocyanin was effective in inhibiting the growth of the fungus *C. albicans* than *A. flavus* i.e., with a zone of inhibition of 25.2 mm and MIC and MKC values were 0.4 and 0.8 mg/ml respectively.

Table 1: MIC and MBC of purified anthocyanin of *C. infortunatum* using the tube dilution assay MRSA–multiple resistant *S. aureus*. mean±SD, *p<0.05*

Organism	MIC	MKC
E. coli	1.6±0.02	3.2±0.38
P. aeruginosa	0.8±0.09	1.6±0.29
E. faecalis	3.2±0.21	6.4±1.5
S. aureus	0.4±0.26	0.8±0.076
MRSA	1.6±0.09	3.2±0.24
A. flavus	1.6±0.06	3.2±2.5
C. albicans	0.4±0.01	0.8±0.061
Tetracycline	0.2±0.02	0.4±0.04
Gentamicin	0.4±0.01	0.8±0.09
Vancomycin	0.8±0.02	1.6±0.08
Amphotericin	0.8±0.05	1.6±0.01

Results of agar diffusion method revealed a significant dose-dependent growth inhibitory activity against the Gram-positive bacteria *S. aureus* meanwhile, *E. faecalis* was found to be more resistant. With Gram-negative strains, only *P. aeruginosa* was found more sensitive. The zones of growth inhibition on *S. aureus* (Gram-positive) and *P. aeruginosa*, (Gram-negative bacteria), were maximum. Optimal zone of inhibition was noticed with *E. coli* compared to *E. faecalis.*

Table 2: Antimicrobial activity of purified anthocyanin of *C. infortunatum* determined by disk diffusion assay, mean±SD, *p<0.05*

Conc. (mg/ml)	Inhibition zone diameter (mm)						
	E. coli	*P. aeruginosa*	*E. faecalis*	*S. aureus*	MRSA	*A. flavus*	*C. albicans*
1.0	2.46±0.31	6.15±0.34	1.93±0.98	9.08±0.4	7.6±0.67	2.74±0.43	10.4±0.21
1.5	7.51±0.43	10.35±1.90	4.39±0.98	11.9±0.69	9.8±0.55	6.75±0.30	12±1.4
2.0	10.2±0.69	13.2±0.3	6.44±0.77	14.6±0.74	12±0.04	10.3±0.95	14.6±-0.32
2.5	12.5±0.1	18.83±2.95	8.12±0.95	21.6±0.74	17±0.94	14.3±0.45	19±0.16
3.0	16.8±0.4	22.5±0.32	10.86±0.4	26.5±0.95	20.8±0.21	18.6±0.32	25.2±0.92

The minimum biocidal concentration of anthocyanin was determined by incubating different concentrations of extracts with a standard inoculum of microbial cultures. After 24 h of incubation, an aliquot was removed from each test samples and the dilutions were plated on agar plates to determine the presence/absence of microbial colonies. The table 1 and 2 shows the antimicrobial activity of anthocyanin was comparable to the bactericidal effect of antibiotics. *C. infortunatum* has been used for centuries in India to counter the infective agents and also for other skin-borne medical ailments.

Table 3: Concentration of k+(μg/ml) in solution of selected bacteria after 1 h of exposure to MIC of anthocyanin. mean±SD, *p<0.05*

Pathogens	K+(μg/ml)
Staphylococcus aureus	0.78±0.001
P. aeruginosa	0.62±0.002
MRSA	0.50±0.001
Escherichia coli	0.38±0.005
Enterococcus faecalis	0.29±0.007

Table 4: Permeability of bacteria to propidium iodide (pi) after 1 h of exposure to anthocyanin at their MIC mean±SD, *p<0.05*

Pathogens	Permeability to propidium iodide (%)
Staphylococcus aureus	95±1.8
P. aeruginosa	80.7±2.4
MRSA	63.7±4.7
Escherichia coli (-)	50.5±0.74
Enterococcus faecalis (+)	37.8±0.99

Mean values±SD for at least three replicates are illustrated

Effects of anthocyanin on intracellular potassium leakage

The K+leakage determination is used to identify alterations of the cell membrane permeability. The effects of anthocyanin on K+release from bacterial strains are shown in table 3. 0.78±0.001 loss of intracellular K+was observed for *Staphylococcus aureus* cells with anthocyanin, at the tested concentration. For *Enterococcus faecalis*, K+ leakage was found as 0.29±0.007 (table 3).

Effects of anthocyanin on bacterial membrane integrity

The integrity of cell membranes can be assessed based on the ability of PI to penetrate the cytoplasmic membrane. PI only penetrates cells with damaged membrane. In this way, the potential of selected EOs components to interfere with membrane integrity after 1 exposure was analyzed (table 4). The PI uptake results suggest that anthocyanin compromise the integrity of the cytoplasmic membrane of both bacteria (*p*<0.05). For *Staphylococcus aureus* the percentage of cells stained with PI after 1 h of treatment (at corresponding MIC) was 95±1.8%. For MRSA exposed to anthocyanin, the damage in the cytoplasmic membrane was about 63.7±4.7% of the total cells (table 4). Sun *et al.,* [16] analyzed the possibilities of multidrug resistance (MDR) that cause antibiotic resistance including targ*et al.* teration, drug inactivation, decreased permeability and increased efflux, drug extrusion by the multidrug efflux pumps serves as an important mechanism of MDR. In the present study, anthocyanin effectively alters the membrane permeability and therefore scope for further analysis among MDR strains.

The major metabolic reactions linked with microbicidal potentialities are glucorination, methylation and sulfation. Polyphenols are proven antimicrobial agents against human pathogens and have been intensively characterized to design novel healthy food ingredients as well as in medical and pharmaceutical fields. Meanwhile, only meager information is available about the microbicidal potentialities of the pure anthocyanins. However, there were a few reports about anthocyanin profiles of different berries and their antimicrobial potentialities with their identified compounds. Commonly, anthocyanins are active against many human microbes, however; Gram-positive bacteria are usually more sensitive to the anthocyanin than Gram-negative bacteria. The plausible mechanisms underlying anthocyanin activity may include both membrane and intracellular interactions of their functional groups. Microbicidal potentialities of fruits and other polyphenol-containing berries are likely to be caused by multiple modes of actions. Anthocyanins contain diverse molecules including weak organic acids, phenolic acids and glycosides of different chemical forms [17].

Alberto *et al.* [18] experiments showed a direct relationship between the phenolic compounds of plant extracts and the antimicrobial power. Fruits of *Aronia melanocarpa* possess rich plant sources of phenolic substances, mainly anthocyanins–glycosides of cyanin. Anthocyanins are water-soluble pigments accounting for their dark blue color in the fruits [2, 3]. They reported that anthocyanin content of berry fruits varies from 7.5 mg/100 g fresh fruit in red currant to 460 mg/100 g fresh fruit in *Aronia melanocarpa*. Cisowska *et al.*, [19] confirmed that anthocyanins were active against various human pathogens, especially Gram-positive bacteria. The present results also showed that some Gram-positive bacteria and Gram-negative bacteria are more susceptible to purified anthocyanin. It is possible that such an effect could be due to anthocyanins. Liepiṇa *et al.*, [20] analyzed the antimicrobial activity of crude anthocyanin extracts from fruits of *Aronia melanocarpa* and *Sorbus aucuparia*. Cisowska *et al.*, [19] reviewed anthocyanins as antimicrobial agents of natural plant origin. Burdulis *et al.*, [21] compared anthocyanin composition, antimicrobial and antioxidant activity in bilberry and blueberry fruits. Liegiutė *et al.*, [22] analyzed the composition of anthocyanin and its antimicrobial activity of sour cherry fruit extracts. Yoon *et al.*, [23] analyzed anti-inflammatory and antimicrobial effects of anthocyanin extracted from black soybean on chronic bacterial prostatitis rat model. The antibactericidal activity of purified anthocynain of the present study against multi-resistant pathogens such as *S. aureus* (MRSA) and *P. aeruginosa* requires special attention. Srirangaraj *et al.*, [24] analyzed multidrug-resistant

Acinetobacter baumannii from nosocomial urinary tract infection. Radhika and Mohaideen [25] analyzed the phytochemicals by fourier transform infrared analysis of *Ulva lactuca* and *Gracilaria corticata* and their effect on antibacterial activity. All these results substantiate the present result of *C. infotunatum* anthocyanin as microbicidal.

CONCLUSION

Calli initiation and growth were best with leaf explants on MS medium supplemented with 0.5 mg/l 2, 4-D+2.0 mg/l BAP. But, the highest anthocyanin yield was obtained on MS medium added with 1.0 mg/l 2,4-D+2.0 mg/l KIN. The result showed that the calli growth and anthocyanin synthesis are inversely correlated. Cyanidin 3-O-sambubioside, delphinidin 3-O-sambubioside and malvidin 3-O-glucoside, delphinidin 3-O-glucoside, cyanidin 3-Oglucoside and petunidin 3-O-glucoside were minors. Malvidin 3-O-glucoside and petunidin 3-O-glucoside are the major anthocyanin noticed in the present study. This clearly shows an optimization of anthocyanins production by cells culture. Further, the study confirms the potentiality of anthocyanin as an antimicrobial against selected pathogens from bacteria and fungi and the results were comparable with the standards. Future studies are warranted to analyze the molecular mechanism of action of anthocyanin against the microbes.

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AUTHORS CONTRIBUTIONS

All the author have contributed equally

CONFLICTS OF INTEREST

There are no conflicts of interest

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