**Abstract**

Tribal people use the flower extract of *Caesalpinia pulcherrima* to cure liver, stomach and skin disorders in Indian traditional medicine. This study aimed to evaluate the protective roles of purified quercetin extracted from suspension culture of *C. pulcherrima* against selected bacterial and fungal pathogens. A simple protocol was developed for callus production using leaf explants. 2, 4-D (2.5 mg/l), BAP (2.5 mg/l) + kin (1 mg/ml) was effective for optimal callus induction. Subsequently, cell suspension culture was established. Role of effect of elicitors in cell suspension culture was carried. Sucrose, ABA and salicylic acid (SA) at different concentrations influenced cell biomass and quercetin accumulation. Cells cultured in the medium fortified with 45 g/L sucrose without ABA/SA showed the highest quercetin content (16.5 mg/g). Flavonoids was purified, fractionated by HPLC-DAD followed by NMR revealed the presence of quercetin, isoquercetin, quercetin, rutin, quercetin 3-O-β-D-xiloside, quercetin 3-O-arabinopyranoside, quercetin 3-O-α-arabinopyranosyl (1 2) β-galactopyranoside, isorhamnetin 3-O-rutinoside and an unknown compound. Subsequently, anthocyanin was evaluated for antimicrobial activity against selected Gram-positive bacteria (*S. aureus, Bacillus subtilis* and *Enterococcus faecalis*), Gram-negative bacteria (*E. coli, and Pseudomonas aeruginosa*) and fungi such as *Aspergillus flavus, Candida albicans* and *Trichophyton rubrum*. Quercetin was found to be active against four bacteria and the fungi- *Candida albicans*. The highest inhibitory effects were found on *S. aureus* and *Enterococcus faecalis*. Gram negative bacteria showed more resistance i.e., with insignificant MIC and MBC values. Among the fungi, *Aspergillus flavus* and *Trichophyton rubrum* displayed remarkable MIC and MBC values. These results suggest that quercetin may be used as a natural antimicrobial agent. Future works are designed to trace the molecular mechanism of antimicrobial potentiality of quercetin against these tested pathogens.

**Keywords:** Antimicrobial, *Caesalpinia pulcherrima*, Quercetin, Suspension culture

**Introduction**

Currently, there are increasing reports on polyphenolic compounds in plant species linked with their biological properties. Phenolic acids including benzoic and cinnamic acid derivatives have been considered as the most common. Among benzoic acid derivatives, p-hydroxybenzoic, protocatechuic, gallic, vanillic and syringic acids were identified in many medicinal plants [1]. The fractionation of cinnamic acid and its derivatives such as p-coumaric, o-coumaric, caffeic, ferulic and chlorogenic acids was also documented. Similarly, flavonoids such as quercetin, rutin and chrysin and tannins like ellagic acid were also reported [2]. *In vitro* and epidemiologic studies suggest that consumption of plant products rich in phenolic compounds may significantly reduce the risk of secondary health problems due to their antioxidant, antimutagenic, anti-inflammatory and antibacterial properties [3].

Plant extracts are natural antimicrobial and antioxidant agents and therefore important in food, cosmetic, medical and agrochemical fields [4]. Most of the fruits are rich in nutrients and bioactive substances. Similarly, to avoid or retard oxidative deterioration and unwanted microbial growth, a wide range of food grade synthetic chemicals are added during food preparation, storage, and distribution. The growing interest in the substitution of synthetic food antioxidants and antimicrobial agents by natural ones has fostered research on plant sources and the screening of raw materials for identifying new natural food additives with a broad spectrum of antioxidant and antimicrobial activity. Special attention has been focused on their extraction from inexpensive or residual sources from the agricultural and food-processing industries [5]. *Caesalpinia pulcherrima* (L.) Sw. of Fabaceae an ethnich medicinal herb used by the locals for curing many ailments. Decoction or infusion of the roots, bark, leaves or flowers is used as a purgative.
It may be used as a mouthwash for teeth or gums, a remedy for colds and fevers, or even as a strong abortifacient. The root is astringent, bitter and in larger doses, is used as an abortifacient. It is used in the treatment of diarrhea. The leaves are febrifuge and tonic. The flowers are febrifuge and, when fresh, sudorific. An infusion is used as a treatment for gall bladder problems. An infusion is used as a treatment for gall bladder problems. It is also used to treat urinary tract problems [6]. In this juncture, the aim of the present study was to investigate the chemical composition and the in vitro antimicrobial activity of purified quercetin of in vitro cell suspension culture from Caesalpinia pulcherrima (L.) Sw.

Material and Methods

Plant material

The fresh excised shoot tips and young leaves of Caesalpinia pulcherrima (L.) Sw. were collected from the gardens.

In vitro cell suspension culture

The explants shoot tips and leaf were washed, followed by dipping in mild tween 20 (2%) and then dipped in Bavistin (1%) for 30 min, rinsed thrice with deionized distilled water. It was further surface sterilized using ethanol (70%) followed by HgCl₂ (0.1%) and then again washed with deionized distilled water thrice. Sterilized explants were cultured on full, half strength MS medium fortified with 2, 4- dichloro phenoxy acetic acid (2, 4-D) 0.5-5 mg/L, BAP 1-5 mg/L, kinetin 0.5-2.5 mg/L and also in combination of these. Culturing conditions are maintained under 16/8 h photoperiod at 24 ± 2°C. Regularly, the cells were harvested. The quercetin content was determined from the harvested cells as stated below.

Quantification of flavonoids

Total flavonoid was quantified by the standard protocol.

Extraction, fractionation, and purification of quercetin derivatives

Cell suspension cultures were extracted with methanol at room temperature for 48 h and was filtered and concentrated under reduced pressure to yield a crude extract and was diluted in methanol water to a ratio of 9:1, and then extracted successively with n-hexane, dichloromethane, ethyl acetate and n-butanol. Ethyl acetate and n-butanol fractions were chromatographe graphed separately on amberlite XAD-16 columns (2 m 8 cm). Aqueous methanol solutions (from 0% to 100%, with 10% increments) were used as the mobile phase, and 11 fractions were collected for each extract (fractions EA1–EA11 and B1–B11, for Ethyl acetate and n-butanol, respectively). The fractions were pooled and subjected to Sephadex LH-20 chromatography (30 cm 45 mm) with methanol water (1:1) as the mobile phase. HPLC-DAD (High Performance Liquid Chromatography with Diode Array Detector) was used to analyze the chemical composition. 13C-NMR spectra were run on a Bruker AV NMR instrument equipped with 5 mm 13C operating at 500 MHz with tetra methyl silane (TMS) as an internal standard.

Microbial strains

Staphylococcus aureus ATCC 25923, Enterococcus faecalis ATCC 29212, Bacillus subtilis ATCC6051, Escherichia coli ATCC 8739, Pseudomonas aeruginosa ATCC 9027, and fungi Candida albicans ATCC 10231, Aspergillus flavus, ATCC 16404, Trichophyton rubrum ATCC 28188 were used. Bacterial suspensions were made in brain heart infusion (BHI) broth to a concentration of approximately 10⁶ CFU/ml using standard routine spectrophometric methods. Suspensions of fungi were made in Sabouraud dextrose broth. Subsequent dilutions were made from the above suspensions, which were then used in the tests.

Evaluation of antimicrobial activity

The minimum inhibitory concentration (MIC) and minimum lethal concentration (MLC) values of extracts were determined by micro broth dilution assay. The extracts were twofold serially diluted with 10% DMSO which contains 5-0.2 mg/ml of quercetin. These
Potassium (K+) leakage

The potassium leakage was determined using a flame emission and atomic absorption spectroscopy used for titration in solution [7]. 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 [8]. This is an efficient protocol employed to determine both viable and total counts of bacteria [9]. The BacLight kit is composed of two nucleic acid-binding stains: SYTO 9 and propidium iodide (PI). Bacterial strains were cultured overnight at 30°C in MHB, centrifuged (3772 g, 10 min), and washed once with saline solution (NaCl, 0.85%). Subsequently, bacteria were resuspended in NaCl to obtain an OD600 nm of 0.134 ± 0.02. Then, 1 mL of each cell suspension was maintained in contact with quercetin (at corresponding MIC) for 1 h. Cell suspension with DMSO at 10% and without quercetin was used as controls. Then, bacterial suspensions were diluted (1:10) in NaCl and 300 µL of each diluted suspension was filtered through a Nucleopore (Whatman) black polycarbonate membrane (pore size 0.22 mm) and stained with 250 µL of diluted SYTO 9 and 250 µL of diluted component PI. The dyes were left to react for 15 min in the dark, at C. The membrane was then mounted on BacLight mounting oil, as described in the manufacturer’s instructions. A LEICA DMLB2 microscope with mercury lamp HBO/100W/3 was used to observe stained bacteria, incorporating a color digital camera to acquire images using IM50 software (LEICA) and a 100 oil immersion fluorescence objective. The optical filter combination for optimal viewing of stained mounts consisted in a 480–500 nm excitation filter with a 485 nm emission filter (Chroma 61000-V2 DAPI/FITC/TRITC). The total number of cells (both stains) and the number of PI stained cells (damaged) were calculated using a program path (Scan Pro 5). The total cell number and the number of PI stained cells on each membrane were estimated from minimum counts of > 20 fields of view. The total cells number per field was approximately 50–200 cells.

Measurement of release of 260 and 280 nm absorbing cellular materials

The amount of the release of 260-nm-absorbing compounds in terms of OD from the selected bacterial cells was carried out in aliquots of 2 mL of the bacterial inocula in sterile peptone water (0.1 g/100 ml) added of quercetin MIC concentration at 37°C. At 0, 30 and 60 min time interval of treatment, cells were centrifuged at 3000 x g, and the absorbance of the obtained supernatant was measured at 260 nm using a 96-well plate ELISA reader [10]. Similarly, control was also tested without adding quercetin. The biuret reaction method as described by Ogundare [11] was employed to determine the quantity of protein leaked from the cells of the organisms. Known concentration of protein was prepared as standards using bovine serum albumin. 1 mL of 18 h bacteria grown on nutrient agar were treated with 9 mL of 5 mg/ml concentration of quercetin. After 30, 60, 90 and 120 min of suspension in quercetin, each suspension was centrifuged at 7000 rpm. To 2 mL of each supernatant obtained was 1 mL of 3 N of NaOH and 1 mL of a CuSO4 solution the mixture was shaken and the absorbance was measured using a digital spectrophotometer. Triplicate readings were made for each supernatant. The absorbance of a blank prepared with the extract solution alone was measured. From the values obtained for the standard, calibration curve was plotted and from this curve the protein concentration leaked from the microorganisms were calculated.

Statistical analysis

The results of 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 comparison. The level of significance was set at 95%. The entire analysis was performed with statistical software SPSS 17 (SPSS for Windows; SPSS Inc, Chicago, IL).

Results and Discussion

Total flavonoid content in the flower and leaf extracts of C. pulcherrima were 1.9 and 4.2 mg/g tissue. In vitro callus initiation was carried out with stem tip and leaf explants. Maximum callus induction (93 - 96%) and highest callus proliferation (348 – 1476 mg FW) was observed in half strength MS medium fortified with 2,4-D (2.5 mg/L) + 2.5 mg/L BAP + kin (1.5 mg/L) with 30 g/L sucrose for all the leaf explants compared to shoot tip (Figure 1a,b and c).
In vitro callus induction from leaf explants of C. pulcherima showing green-creamy friable type

Based on the growth index, the cell culture of C. pulcherima could be well maintained with an initial inoculum of 0.5 g of fresh cells inoculated into 20 mL of liquid MS medium contain various concentrations and combinations of 2, 4-D + BAP + kin in 100 mL Erlenmeyer flasks and placed on a rotary shaker at 100 - 120 rpm. 2, 1 and 1.5 mg/L 2, 4-D + BAP + kin respectively displayed remarkable cell suspension culture than any other combinations i.e., 7.66 g FW and the lowest with 0.5 + 0.5 + 0.5 mg/L 2, 4-D + BAP + kin (3.7 g FW) (Figure 2). Generally, plant cell suspension cultures fail to detach completely after division, and forms multicellular aggregates. During subculture, a sterilized stainless steel sieve with 850 μm pore size was employed to separate small cell clumps from the larger clumps. Smaller clumps were selected for the quercetin analysis because as cell aggregates enlarge, cells seated in the interior of a large cell clumps are not properly exposed to nutrients, light, oxygen, and other micro-environmental factors compared to younger peripheral cells. These physico-chemical gradients in turn alter cell proliferation and phytochemical production. The cells cultured in MS medium fortified with 1 mg/L BA along with 1 mg/L 2,4-D and 1 mg/L kin exhibited the lowest quercetin content (1.24 mg/g), whereas cells cultured in half MS medium fortified with 2 mg/L 2, 4-D + 1 mg/L BAP + 1.5 mg/L kin produced the maximum quercetin content of 6.98 mg/g DW on 24 th day. The amount of quercetin increased proportionally with the growth phase of the suspension culture.
Different sucrose concentration influenced the cell biomass and quercetin synthesis in cell suspension cultures of *C. pulcherrima*. However, cultures without sucrose (control) showed cell death. Optimal dried cell weight was noticed from the cells cultured in the medium fortified with 45 g/L sucrose as compared to medium fortified with 15 g/L i.e., 10.5g FW and 167.5 mg/g quercetin content. Meanwhile, when the level of sucrose was further enhanced (60 g/L or more), there was a reverse trend in cell growth was noticed. The amount of quercetin produced in the cells cultured in medium fortified with 45, 60 and 75 g/L sucrose achieved remarkable level i.e., 16.5-3.9 mg/g, each of which was significantly different from the other experimental cell cultures sets. Among these three treatment, culture fortified with 45 g/L sucrose showed the highest growth (10.4 g FW) with optimal quercetin content, hence the addition of 45 g/L sucrose into the cell culture of *C. pulcherrima* could be used for increasing quercetin level. The relation between quercetin content and sucrose concentration may be due to the osmolytic property of sugar i.e., the increased sucrose content leads to exosmosis in the cell cultures.

*C. pulcherrima* in cell suspension cultures (3.4 mg/g DW) on 18th day which showed a significant accumulation on 24th day under physical elicitation by UV light. Meanwhile, light (24 h) or dark (24 h), peptone water and yeast treatments results no significant changes in the quercetin level in the culture medium. The addition of phenylalanine as precursor at lower dose enhanced quercetin content of cells in the plants, which ranged from 3.7 – 6.8 mg/g DW. Chemical elicitors, which include GA3, ABA and SA at different concentrations, were successful in inducing quercetin biosynthesis in cell suspension cultures (6-10.6 mg/g DW). Zinc sulphate at lower dose showed slight rise in quercetin accumulation (5-6 mg/g DW), but the content was lower than that of SA or ABA treatments. MeJ did not showed remarkable increase in quercetin production in cell cultures. Regardless of the parameters, the most promising effect on quercetin synthesis in cell suspension cultures was shown by chemical elicitors, SA (50 μM) or ABA (0.1 mg/l) (14.6 mg/g DW and 12.8 mg/g DW, respectively).

The resulted quercetin was purified and fractionated by HPLC-DAD revealed a pool of compounds (Figure 3).

![Figure 3. HPLC-DAD of purified flavonoids of *C. pulcherrima*](image)

Subsequently, 13C-NMR analysis was attempted revealed the spectrum with carbonyl group at 176.2 ppm and aromatic carbon group from 93.8-164.3 ppm (Figure 4). The corresponding 13C NMR peak positions for isolated compound were showed resemblance with the pure quercetin which was also confirmed by previous literatures. 93.8, 98.6, 103.4, 115.4,116.0 (Ar-C), 120.4, 122.4, 136.1, (Ar-C), 143.5, (Ar-C), 147.2, (Ar-C), 148.1, (Ar-C), 156.6, (Ar-C), 161.1, i.e, (Ar-C), 164.3, (Ar-C) and 176.2, (Ar-C=O). Thus, it can be confirmed that the isolated compound is found to be quercetin. Structural characterization of purified compounds using NMR analysis showed the presence of nine compounds which includes, quercetin, isoquercetin, quercetin, rutin, quercetin 3-O-β-D-xyloside (Reinutrin), quercetin 3-O-arabinopyranoside (Guajaverin), quercetin 3-O-u-arabinopyranosyl (1 2) β-galactopyranoside, and Isorhamnetin 3-O-rutinoside (Narcissoside) (Figure 4).
Antimicrobial potential

The purified quercetin from *C. pulcherrima* was screened for their microbicidal activities against Gram-positive bacteria (*S. aureus, Bacillus subtilis, and Enterococcus faecalis*), Gram-negative bacteria (*E. coli, and Pseudomonas aeruginosa*) and fungi such as *Aspergillus flavus, Candida albicans* and *Trichophyton rubrum*. Table 1 displayed that quercetin of *C. pulcherrima* was active against four bacteria and a fungi *Candida albicans*. The highest inhibitory effects were found against *S. aureus* and *Enterococcus faecalis*. Comparatively, Gram negative bacteria showed a range of 0.125 – 0.25 mg/ml MIC and 0.25 – 0.50 mg/ml MBC values. The zones of inhibition (mm) displayed no remarkable differences among the tested pathogens i.e., 9.78 to 28.5 mm (Table 1). These variations may be associated with the varied structural features of cell surface between Gram-negative and Gram-positive bacteria [12]. *Aspergillus flavus* and *Trichophyton rubrum* also displayed optimal MIC and MKC and the values are at par with the antifungal properties of the leaf extract of *Aloe vera* extracted with pure methanol and ethanol [13]. The MIC and MKC values are presented in Table 1.

**Table 1.** Minimum inhibitory concentration (MIC) and Minimum killing concentration (MKC) of the purified quercetin from *C. pulcherrima*

| Pathogens                  | Zone of inhibition (mm) | MIC (mg/ml) | MBC/ MKC (mg/ml) |
|---------------------------|-------------------------|-------------|------------------|
| *Staphylococcus aureus* (+)| 28.5 ± 0.97             | 0.0625      | 0.125            |
| *Bacillus subtilis* (+)   | 13.2 ±0.36              | 0.75        | 1.5              |
| *Escherichia coli* (+)    | 17.8 ± 0.54             | 0.25        | 0.50             |
| *Enterococcus faecalis* (+)| 22.5 ± 1.3             | 0.125       | 0.250            |
| *Pseudomonas aeruginosa* (-)| 23.2 ± 0.22          | 0.125       | 0.250            |
| *Aspergillus flavus*      | 16.9 ±0.78              | 0.25        | 0.50             |
| *Candida albicans*        | 23.3 ± 2.1              | 0.125       | 0.250            |
| *Trichophyton rubrum*     | 9.78 ±0.02              | 2.5         | 5.0              |
| Tetracycline              | 32 ±1.8                 | 0.0625      | 0.125            |
| Fluconazole               | 31.6 ± 0.82             | 0.0625      | 0.125            |

Fernandez-AgulloA [14] revealed that the microbicidal activities of herbal extracts may reside in a variety of diverse molecules, such as phenolic acids, flavonoids, tannins, alkaloid, terpenoid, sotfooxide and lactone. It has been reported that gallic acid, vanillin, eugenol, eugenin, acetyl eugenol, quercetic acid in the clove bud extracts were effective against *L. monocytogenes, S.aureus and Salmonella* [15]. The presence of quercetagetin, patuletin, kaempferol and quercetin glycosides from *Anthemis cotula* flowers displayed high
bactericidal activity against both Gram-negative and Gram-positive microorganisms [16]. Caffeic acids in tarragon and thyme were effective against viruses, bacteria, and fungi [17]. *S. aureus* and *E. coli* exhibited dose dependent and susceptibility (*P < 0.01*) when exposed to 4 mg/ml of the tannin monomers from eight different woody plant species extracts [18]. Phenolic compounds in the plant had contributed to the antimicrobial properties, and the antimicrobial capacity was dependent on the action mode of polyphenols, source and concentration of phenolic compounds, extraction methods, varieties and microorganism species [17, 19, 20]. In addition, there are the synergistic effect of all kinds of polyphenolic compounds on bacterial stains and fungal species [21].

### Effects of quercetin 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 4. 0.23 ± 0.004 loss of intracellular K+ was observed for *B. subtilis* cells with anthocyanin, at the tested concentration. For *S. aureus*, K+ leakage was found as 0.72 ± 0.009 (Table 2).

### Effects of quercetin on bacterial membrane integrity

The integrity of cell membranes can be assessed based on the ability of PI to penetrate cytoplasmic membrane. PI only penetrates cells with damaged membrane. In this way, the potential of quercetin to interfere with membrane integrity after 1 h exposure was analyzed (Table 2). The PI uptake results suggest that quercetin compromises 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 93.7%. For *Bacillus subtilis* exposed to quercetin the damage in cytoplasmic membrane was about 44% of the total cells (Table 2).

### Effect on Release of 260 and 280 nm absorbing molecules

The release of cellular molecules like nucleic acids and proteins from the bacteria treated with quercetin was presented in the table 3. When the bacteria were treated with the MIC concentration of quercetin, there was a continual increase in the concentration of 260 nm absorbing materials over 120 min of incubation. The control treated with DMSO did not show an increase in the concentration of 260 nm absorbing materials after 60 min of incubation. The significant increase in the OD at 260 nm noticed in the bacterial solution treated with quercetin, indicating active leaching of intercellular materials like nucleic acids to the outer solution by cellular leakage.

### Table 2: Concentration of K+ (µg/mL) in solution and permeability of bacteria to propidium iodide (PI) after 1 h of exposure to quercetin at their MIC values

| Pathogens                        | K+ (µg/mL) | Permeability to propidium iodide (%) |
|----------------------------------|------------|-------------------------------------|
| *Staphylococcus aureus* (+)      | 0.72 ± 0.009 | 93.7 ± 2.6                           |
| *Bacillus subtilis* (+)          | 0.23 ± 0.004 | 44 ± 0.78                            |
| *Escherichia coli* (-)           | 0.31 ± 0.007 | 62.8 ± 3.8                           |
| Enterococcus faecalis (+)        | 0.51 ± 0.003 | 74.5 ± 1.7                           |
| *Pseudomonas aeruginosa* (-)     | 0.49 ± 0.002 | 82.5 ± 0.91                          |
| Tetracycline                     | 0.87 ± 0.01  | 97.8 ± 4.9                           |

### Table 3: Leakage of 260 molecules after exposure to quercetin at their MIC values. Time slot 0 to 120 minutes.

| Pathogens                        | 0 ± 0.83 | 30 ± 0.33 | 60 ± 0.23 | 90 ± 0.13 | 120 ± 0.03 |
|----------------------------------|----------|-----------|-----------|-----------|------------|
| Control                          | 1 ± 0.22 | 1 ± 0.06  | 1 ± 0.12  | 1 ± 0.28  | 1 ± 0.33   |
| *Staphylococcus aureus* (+)      | 1 ± 0.97 | 1.87 ± 0.3 | 3.5 ± 0.44| 5.1 ± 0.2 | 5.4 ± 0.3  |
| *Bacillus subtilis* (+)          | 1 ± 0.06 | 1.17 ± 0.02| 1.7 ± 0.09| 2.7 ± 0.08| 2.9 ± 0.02 |
| *Escherichia coli* (-)           | 1 ± 0.29 | 1.3 ± 0.01 | 2.1 ± 0.02 | 3.2 ± 0.01| 3.5 ± 0.27 |
| Enterococcus faecalis (+)        | 1 ± 0.3  | 1.47 ± 0.11| 2.6 ± 0.28| 3.9 ± 0.31| 4.2 ± 0.36 |
| *Pseudomonas aeruginosa* (-)     | 1 ± 0.07 | 1.59 ± 0.24| 3 ± 0.09 | 4.6 ± 0.05| 4.8 ± 0.12 |
| Tetracycline                     | 1.17 ± 0.04| 3.5 ± 0.01| 4.7 ± 0.27| 5.2 ± 0.35| 6.3 ± 0.41 |

The permeability of the cytoplasmic membrane was significantly impaired by the application of quercetin into the cell culture. Hydrophobicity may be the mode of action that leads to the accumulation of quercetin inside bacterial cell membranes, resulting in cellular lysis by disturbance of their structure and increased cellular leakage. However, no marked changes in the
OD of control cells of test pathogens were seen during the study. After 60 min of treatment, approximately more than 2.5 fold increases was noticed in the OD of the bacterial cell culture filtrates treated with quercetin. Similarly, quercetin facilitated the leakage of protein more soundly in *S. aureus* i.e., 1.2, 1.49, 1.67 and 1.7 mg/ml of protein were leaked at 30, 60, 90 and 120 min interaction. The trend of protein leakage by quercetin from tested bacteria further substantiates the disruption of cell membrane (Table 4). Thus the entire study reveals that quercetin exerted its inhibitory action through membrane permeabilization associated with membrane-disrupting leading to simultaneous reduction in cell viability, loss of 260, 280 nm absorbing materials, leakage of potassium ions, which confirms the loss of membrane integrity.

### Table 4: leakage of protein (mg/ml) after exposure to quercetin at their MIC values. Time slot 0 to 120 minutes.

| Pathogens                  | 0     | 30     | 60     | 90     | 120    |
|----------------------------|-------|--------|--------|--------|--------|
| Control                    | 0.22±0.03 | 0.23±0.01 | 0.22±0.02 | 0.22±0.08 | 0.22±0.03 |
| *Staphylococcus aureus* (+)| 0.25±0.01 | 1.2±0.05 | 1.49±0.04 | 1.67±0.21 | 1.7±0.33 |
| *Bacillus subtilis* (+)    | 0.24±0.06 | 0.44±0.06 | 0.57±0.02 | 0.63±0.02 | 0.7±0.03 |
| *Escherichia coli* (-)     | 0.23±0.09 | 0.68±0.04 | 0.97±0.001 | 1±0.01   | 1.1±0.07 |
| Enterococcus faecalis (+)  | 0.22±0.3  | 0.85±0.11 | 1.0±0.29  | 1.2±0.31  | 1.3±0.36 |
| *Pseudomonas aeruginosa* (-)| 0.21±0.05 | 0.9±0.22  | 1.2±0.05  | 1.4±0.01  | 1.5±0.12 |
| Tetracycline               | 0.26±0.04 | 1.5±0.04  | 1.7±0.07  | 1.8±0.05  | 1.9±0.09 |

Cetin-Karaca and Newman [21] analyzed antimicrobial efficacy of natural phenolic compounds against Gram positive food borne pathogens. Chun et al., [22] validated the phenolic antioxidants from clonal oregano with antimicrobial activity against *Helicobacter pylori*. Nohynek et al., [23] evaluated the berry phenolics with antimicrobial properties and elucidated its mechanisms of action against severe human pathogens. Xiaoyong and Luming [24] screened leaf phenolic constituents, antimicrobial and antioxidant properties of blueberry. All these reports substantiate the present antimicrobial potentials of *C. pulcherima* quercetin.

### Conclusion

Quercetin from *C. pulcherima* was extracted from *in vitro* cell suspension culture and was characterized. The quercetin content was correlated well with antimicrobial activity. Mechanism of activity was by the disruption of cell membrane and that was confirmed by potassium leakage and bacterial membrane integrity assay. This research offers innovative strategies to develop functional foods and supplements against chronic bacterial infections.

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### Conflicts of interest

The authors declared that there is no conflict of interest.

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