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Antimicrobial and antibiofilm potential of *Curcuma longa* Linn. Rhizome extract against biofilm producing *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolates

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Abstract: More than 65% of all human bacterial infection are associated with biofilm. Bacteria in such biofilms are 10 to 1000-fold more resistant to antibiotics than free living bacteria cells. Organisms such as *S. aureus* and *P. aeruginosa* are responsible for a significant number of biofilm related infections. In this study, we investigated the antimicrobial and anti-biofilm activity of *C. longa* L. rhizome extract against biofilm producing *S. aureus* and *P. aeruginosa* isolates. The results of MIC and MBC demonstrated promising antibacterial activity of the rhizome extract. TLC and column chromatography detected various curcuminoids while phytochemical analysis also reveals presence of number of bioactive compounds such as alkaloids, flavonoids, phenolics, terpenoids, etc. Micro titer plate assay indicated significant inhibition of biofilm formation in clinical isolates treated with turmeric extract. Thus, on basis of our results turmeric extracts can be considered as natural antibiofilm and antibacterial agent.

Key words: *Curcuma longa L.*; Turmeric; Anti-bacterial; Anti-biofilm; *S. aureus*, *P. aeruginosa*; Phytochemicals.

Introduction

Turmeric is a spice derived from the rhizomes of *Curcuma longa L.*, belonging to ginger family (*Zingiberaceae*) (1). Its rhizomes are source of a bright yellow dye and spice which are oblongonate, pyriform, and they are house hold remedy in Nepal and is popularly known as 'Besar' (2). As a powder called turmeric, it has been in continuous use for its flavor, color and spices in Indian subcontinent. Turmeric is extensively explained as a medicinal plant in Indian material medica (Dravyaguna Sastra) and has been well documented in Ayurveda for its therapeutic potentials and described in Kusthagna (Anti-dermatosis) and Visaghna (Anti-poisonous) (3). Ayurvedic medicine documents it as a treatment for various respiratory conditions such as asthma, bronchial hyperactivity and allergy, as well as for liver disorders, anorexia, rheumatism, diabetic wounds, runny nose, cough and sinusitis (4). Similarly, Chinese medicine also used it for abdominal pain (5). The dried rhizome of turmeric contains a large number of medicinally important phytochemicals known as the curcuminoids (6). This polyphenolic compound has shown wide variety of biological activities from antioxidant, neuroprotective, antitumor, anti-inflammatory, anti-acidogenic, radioprotective to arthritis(7). Among two other curcuminoids, demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC), Curcumin is the best studied active ingredient of turmeric and has been assessed in various clinical trials its therapeutic ability on inflammatory, skin, eye, central nervous system, respiratory, cardiovascular, gastrointestinal, urogenital and metabolic disorders (8, 9).

Biofilms contains group of microorganisms that are adhered at biotic and abiotic surfaces which remain in extracellular compounds released by biofilm cells (10). Process of biofilm formation follows multiple steps such as (i) adsorption of molecules (macro and micro molecules) to surfaces; (ii) bacterial adhesion to the surface and release of extracellular polymeric substances (EPS); (iii) colony formation and biofilm maturation (11). Biofilm related infection grows on natural surfaces such as teeth, heart valves (endocarditis), in the lungs of cystic fibrosis (CF) patients, chronic and secretory otitis media, intravenous catheters, and stents (12). More than 65% of all human bacterial infection are associated with biofilm and there is no available antibiotic that specially target the biofilm since bacteria in biofilm are 10 to 1000-fold more resistant to antibiotics than free living bacterial cells (13, 14). Antibiotics penetrating into these biofilm matrixes are less effective as most of the antibiotics are active only against unattached microorganisms. Ceri et al. (15) reported that biofilm-associated E. coli required greater than 500 times MIC of ampicillin

for a 3-log reduction whereas Williams et al. (16)found that S. aureus biofilms required greater than 10 times MBC of vancomycin for a 3-log reduction. Antibiotics resistance in biofilm are supposed to be due to reasons such as slow or incomplete penetration of the antibiotics into the biofilm, an altered chemical microenvironment within the biofilm and a subpopulation of microorganisms in a biofilm (11). Novel strategies such as isolations of quorum quenching compounds, dispersal of formed biofilms, combinations of antibiotics with quorum quenching compounds are being tried as a measure to counter biofilm infections (17). However, these strategies are yet to get approved for daily use. Furthermore, the misuse of antibiotics has also contributed to development of drug resistance, which might synergize the bacteria infected disease. Therefore, novel strategies other than antibiotics should be developed to combat the bacterial and biofilm formation.

Organisms such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* are usually associated with nosocomial infections and life-threatening chronic infections. Several studies have also demonstrated the involvement of *P. aeruginosa* and S. aureus biofilm for persistent infections (18) (19). *P. aeruginosa* strong pathogenicity and resistance to antibiotic therapy has largely influenced by its inherent biofilm-forming capacity(20). Lately, studies have also shown that the presence of *P. aeruginosa* biofilms is the contributor to wound chronicity and persistence, and appears to delay or prevent the healing of the wound (21). The persistence of chronic *P. aeruginosa* lung infections in CF patients are also reported due to biofilm-growing mucoid (alginate-producing) strains(22). Similarly, *S. aureus* and its methicillin resistant strains are also responsible for a significant number of biofilm-related infections(23).

Many plant-derived natural products possessed antibacterial as well as anti-biofilm functions in vitro. *Curcuma longa L.* extract expressed antibacterial activity against a range of bacteria including *P. aeruginosa* and *S. aureus* (24). The aim of this study was to investigate the antibiofilm activity of Antimicrobial and antibiofilm potential of Curcuma longa Linn. rhizome extract against biofilm producing *S. aureus* and *P. aeruginosa* isolates.

Materials and Methods

Plant materials

The rhizome of *C. longa* L. was purchased from the local market of Sindhuli, Nepal. The collected rhizome was identified by Natural Product Research Laboratory (NPRL), Thapathali, Kathmandu (Voucher number:456). The voucher specimen of this material has been deposited in Natural Product Research Laboratory (NPRL), Thapathali, Kathmandu.

Extraction of turmeric powder

The rhizome of *C. longa L.* was washed thoroughly with distilled water and was cut into small pieces and shade dried for a month. After complete drying, the rhizome of turmeric was reduced to powder form with the help of electric grinder. Twenty grams of turmeric powder was packed in Soxhlet apparatus for extraction from the rhizome by the use of 250 ml of ethanol. The extracts were filtered using Whatman No. 1 filter paper, and the filtrate were concentrated with the help of rotatory evaporator under reduced pressure. The concentrated extract was stored at 4 °C, protected from light and humidity for further analysis.

Bacterial strains

The bacteria used were *S. aureus* and *P. aeruginosa* clinical isolates. These clinical isolates were collected from Manmohan Memorial Medical College & Teaching Hospital, Kathmandu.

Silica gel column chromatography

Ethanolic extract was subjected to Column chromatography in silica gel glass column. About 1gm of crude turmeric extract were mixed with 1 ml methanol and loaded on to the column (34×1.5cm) and eluted with Chloroform**:** Methanol followed by methanol with increasing polarity. All the collected fractions were subjected to TLC (25).

Separation of curcuminoids by TLC

Using a capillary, a small spot of solution containing *C. longa* extract sample is applied to a plate, about 1.5 centimeters from the bottom edge. Here, slurry of silica gel acts as stationary phase and chloroform: methanol in the ratio of 19:1 acts as mobile phase. Then, mobile phase moves up to the plates by capillary action, meets the sample mixtures. Different compounds in the sample mixture travel at different rates due to the differences in their attraction to the stationary phase and because of differences in solubility in the solvent(25).

Phytochemical screening of turmeric extract

Phytochemicals screening was carried out ethanolic extracts of *C. longa* using standard procedure (26). Each extract was subjected to the test for glycosides, alkaloids, carbohydrate, flavonoids, saponins, phenols and tannins.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

To determine minimum inhibitory concentration (MIC), broth dilution method was used (27). Two-fold serial dilution of the aqueous extract of turmeric were made with LB broth at final concentrations of 80,40, 20,10, 5, 2.5 and 1.25mg/ml. Standardized bacterial culture of *S. aureus* and *P. aeruginosa* was inoculated (0.1ml) into the dilutions and incubation was done at 37 °C for 24 hours. The lowest concentrations which showed no turbidity was recorded as the MIC of the extracts.

Minimum bactericidal concentration (MBC) was also monitored to find out whether the bacterial strains were actually killed or their growth was merely inhibited. For this, the content of the tubes showing MIC and tubes with concentrations greater than MIC were spread on separate LB agar plates and incubated at 37 °C for 24 h. The lowest concentration showing no growth on the plate was recorded as MBC.

In vitro biofilm formation assay

This quantitative test described by Christensen et al

is considered the gold-standard method for biofilm detection (28). Organisms isolated from fresh agar plates were inoculated in 5 mL of L.B broth with 1% glucose. Broths were incubated at 37 °C for 24 h. The cultures were then diluted 1:100 with fresh medium. Individual wells of sterile 96 well flat bottom polystyrene microtiter were filled with 200 µL of the diluted cultures. The control organisms were also incubated, diluted and microtiter plate. Negative control wells contained inoculated sterile broth. The plates were incubated at 37 °C for 24 hrs. After incubation, contents of each well were removed by gentle tapping. The wells were washed with 0.2 mL of phosphate buffer saline (pH 7.2) four times. This removed free floating bacteria. Biofilm formed by bacteria adherent to the wells were stained by crystal violet (0.1%). Excess stain was removed by using deionized water and plates were kept for drying. While cell-bound dye was eluted with 200µl of 33% glacial acetic acid and the absorbance of eluted solution was measured at 578 nm using a micro titer plate reader. The experiment was performed in triplicate and repeated three times. The interpretation of biofilm production was done according to the criteria of Stepanovic et al(26).

Effect of turmeric extract on biofilm formation

The effect of *C. longa* extract on the biofilm formation of bacterial pathogens was determined by quantifying the biofilm biomass through Microtiter Plate assay (27). Briefly, overnight cultures of test pathogens were added into 1 ml of fresh LB medium and cultivated in the presence of *Curcuma longa* extracts at the concentration of (0.5–2 mg/ml) without agitation for 16 h at 30 °C. After incubation, the planktonic cells in Microtiter Plate were removed by rinsing the wells twice with sterile water. The surface-adhered cells in the Microtiter Plate wells were stained with 200µl of 0.2% crystal violet (CV) solution. After 10min, excess Crystal Violet solution was removed by phosphate buffer and Crystal Violet in the stained cells was solubilized with 250µl of 95% glacial acetic acid. The biofilm biomass was quantified by measuring the intensity of CV at OD 578 nm using micro titer plate reader(29).

Statistical analysis

Each sample analysis was performed in triplicate. All results presented are means (±SEM) of at least three independent experiments. Statistical analysis was performed with GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA).

Table 1. Screening of phytochemical constituents of *C. longa* extract.

Phytochemical	Present / Absent
Alkaloids	+
Anthraquinone	+
Carbohydrate	+
Cardiac glycosides	+
Terpenoid	+
Phenolic	+
Saponin	-
Tannins	-

Results

Phytochemical Screening

Phytochemical screening showed presence of a number of bioactive compounds such as alkaloids, anthraquinones, glycosides, flavonoids, phenolics and terpenoids while secondary metabolites such as saponins and tannins were absent in turmeric as shown in Table 1. Similarly, the extractive value of turmeric extract is found to be 12.74%.

Column chromatography and TLC

Different active constituent of turmeric powder such as curcumin, Bisdemethoxycurcumin and demethoxycurcumin were successfully detected directly from the ethanolic extract of *C. longa* by column chromatography and TLC. When a turmeric extract was separated on TLC plate each band produced molecular ion peak corresponding to curcumin, Bisdemethoxycurcumin and Demethoxycurcumin as shown in table 2. Each plate was developed to a height of about 5.8cm using mobile phase chloroform: methanol in the ratio of 19:1. Column chromatography of ethanolic extract using chloroform and methanol mobile phase show the separation of 3 constituent band.

Determination of MIC and MBC of turmeric extract

The results of minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) indicated that turmeric extract had the ability to inhibit and completely kill the bacterial strains used in this study. *C. longa* was able to inhibit the growth of *S. aureus* at the concentration of 10 mg/ml (MIC) and

Table 2. Separation of curcuminoid by TLC and column chromatography.

TLC mobile phase	Ratio	RF value		
		Curcumin	DMC	BDMC
Reference	19:1	0.75	0.55	0.39
Sample (chloroform: methanol)	19:1	0.724	0.551	0.27

DMC=Demethoxy curcumin, BDMC= Bisdemethoxy curcumin.

Table 3. MIC and MBC values of turmeric extract against tested bacteria.

Bacterial stain	MIC	MBC
Staphylococcus aureus	10mg/ml	20mg/ml
Pseudomonas aeruginosa	40mg/ml	80mg/ml

at the concentration of 20 mg/ml, recorded as MBC, no growth was found on LB agar whereas *P. aeruginosa* exhibited no visible growth at the concentration of 40 mg/ml (MIC) and there is no growth found at the concentration of 80 mg/ml (MBC) and the values are summarized in Table 3.

Screening of biofilm producer among *staphylococcus aureus* and *Pseudomonas aeruginosa*

A total of 19 *P. aeruginosa isolate* and 20 *S. aureus* were included in the present study. All isolates were subjected to inoculate on microtiter plate. Out of 19 *P. aeruginosa* isolates, 10 were biofilm producers *i.e.* 52.63% whereas 9 isolates of *P. aeruginosa* were non biofilm producers *i.e.* 47.36%. Among biofilm producer isolate, 30% of strain showed the ability to form strong biofilm, 40% of strain showed moderate and 30% of strain showed weak biofilm formation in microtiter plate as shown in Fig. 1.

Out of 20 *S. aureus,* 11 isolates were biofilm producers *i.e.* 55% whereas 9 isolates were non biofilm producers *i.e.* 45%. Among biofilm producer isolate, 70% of strain showed the ability to formed strong biofilm, 20% of strain showed moderate and 10% of strain showed weak biofilm formation in microtiter plate. The OD Biofilm producer of *S. aureus* and *P. aeruginosa* isolates at 578nm is illustrated in Fig 2.

Evaluation of anti-biofilm activities of ethanolic turmeric extract

In biofilm formation assay, a concentration dependent decrease in biofilm formation was observed in *P. aeruginosa* and *S. aureus* isolates with the ethanolic turmeric extract. The extract showed 26.756–58.55%, and 48.862–77.72% inhibition in biofilm formation of *P. aeruginosa* and *S. aureus* respectively at the concentration of 0.5–2 mg/ml (Table 4, Fig 3).

Discussion

Various biological activities of plant extracts are believed to be due to the presence of bioactive compounds (30). These plant secondary metabolites are nutritional constituents which are present in very tiny amounts in plants and have the potential for influencing the physiological and cellular activities after consuming them. As a popular spice, Turmeric, rhizome of *C. longa,* have shown anti-microbial activities against different bacteria, viruses, fungi, and parasites(31). In this context, we investigated antimicrobial and antibiofilm activity of *C. longa* Linn. rhizome extract against biofilm producing *S. aureus* and *P. aeruginosa* isolates.

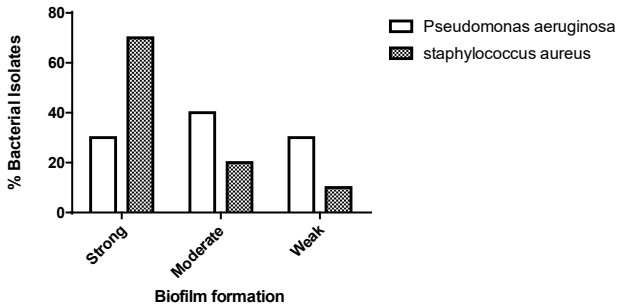

Figure 1. Classification of bacterial isolate according to their ability to form biofilm.

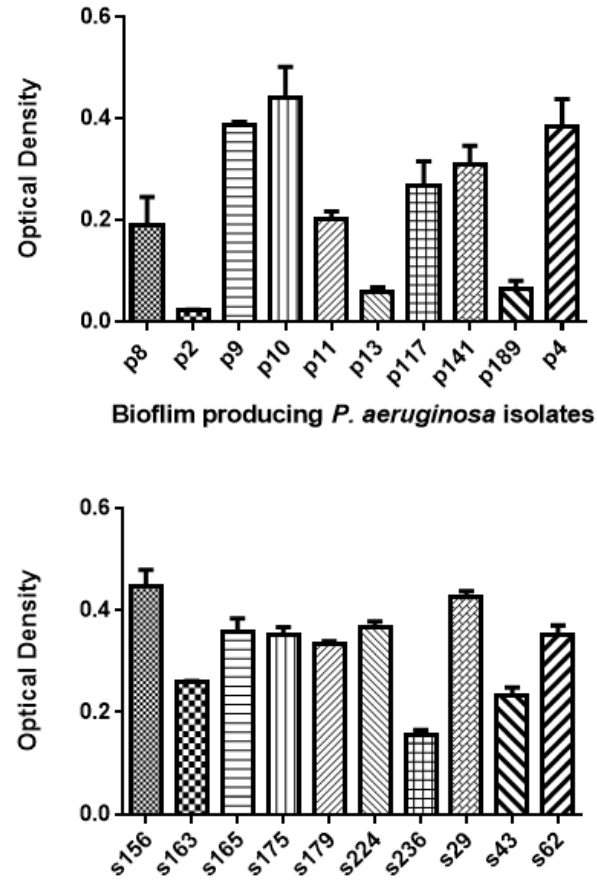

Figure 2. Optical density of Biofilm producer of *S. aureus* and *P. aeruginosa* isolates.

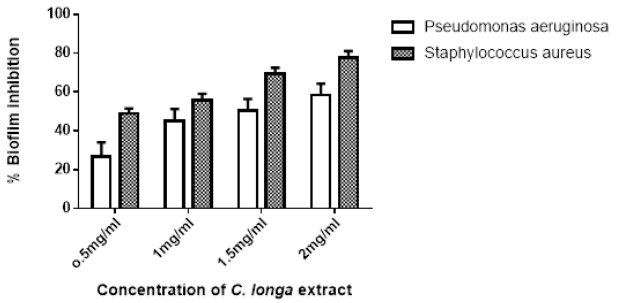

Figure 3. % Biofilm Inhibition of *P. aeruginosa* and *S. aureus* isolates by *C. longa* extract.

The present study indicated significant anti-bacterial activity of *C. longa* extract against two pathogenic bacterial strains. The results of MIC and MBC of *C. longa* extract demonstrated promising antibacterial activity of *C. longa* rhizome. Our observations were confirmed by a similar study of (32) who evaluated antimicrobial activity of extracts of *C. longa* rhizome against four bacterial strains. The MIC and MBC values for different strains of *C. longa* rhizome extract ranged from 4 to 16 g/L and 16 to 32 g/L respectively. A similar finding is attained in a study of anti-microbial activity of *C. longa* rhizome extract, where methanol extract of *C. longa* rhizome showed highest inhibition against *S. aureus* clinical isolates(33). One study also showed that curcumin and various other oil fractions found in tur-

Table 4. Effect of ethanolic turmeric extract at different concentration on biofilm formation.

Concentration of curcuma longa extract	% Biofilm inhibition by Curcuma longa extract in *P. aeruginosa*	% Biofilm inhibition by curcuma longa extract in *S. aureus*
0.5 mg/ml	26.75±7.3	48.86±2.6
1 mg/ml	44.95±6.3	55.81±3.2
1.5 mg/ml	50.57±5.8	69.34±3.07
2 mg/ml	58.55±5.6	77.72±3.32

meric may affect the growth of *P. aeruginosa* by down regulating the expression of virulence factors, biofilm initiation and quorum sensing(34). Turmerone, and curlone presented in Turmeric oil also exhibited excellent antagonistic activity against the bacterial strains such as *P. aeruginosa, B. subtilis, E. coli and S. aureus*(35).

Different active constituent of turmeric such as curcumin, Bisdemethoxycurcumin and demethoxycurcumin were successfully detected from the ethanolic extract of *C. longa* by column chromatography and TLC using reported RF values(25). The pharmacological activity of turmeric has been attributed mainly to these curcuminoids consists of curcumin (CUR) and two related compounds demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC)(36). Typical curcuminoids composition of Indian varieties was found to be in the range of CUR 52–63%, DMC 19–27% and BDMC 18–28%(37). These curcuminoids have extensive biological activity as an antioxidant, neuroprotective, antitumor, anti-inflammatory, anti-acidogenic, radioprotective and arthritis(38). Curcuminoids, present in *C. longa*, are also being known for their hypoglycemic property. BDMC reported as an inhibitor to inactivate human pancreatic α-amylase (HPA) can be developed as anti-diabetic compound(39). Curcumin, one of curcuminoids, showed anti-biofilm against two strains of *P. aeruginosa* of two cystic fibrosis patients with MIC values of 16 µg/mL(40). A recent study assessed the effectiveness of curcumin in inhibiting and degrading polymicrobial catheters, where they concluded curcumin as a potential candidate for development of new antibiofilm drugs against polymicrobial catheters(41). A study investing the chloroform extract of turmeric suggested that turmeric could affect multiple cellular activities in biofilm formers exhibiting antibiotic resistance by modulating adherence, EPS production, motility and surface hydrophobicity (42).

Phytochemical analysis showed presence of a number of bioactive compounds such as alkaloids, anthraquinones, glycosides, flavonoids, phenolics and terpenoids. There are reports showing such phytochemicals exerts antibacterial activities against sensitive and resistant pathogens via different mechanisms of action(43). Similarly, evidence also found phytochemicals from nature to have the potential to be developed as preventative agents or therapeutics against biofilm-based infections(44).

Bacteria within biofilms are more resistant to antibiotics and chemical agents than planktonic cells in suspension(45). *P. aeruginosa* and *S. aureus* are two of the most prevalent nosocomial and respiratory pathogens; both often expressing multiple drug resistance. Both organisms adopt a biofilm mode of growth, which contributes to high tolerance to antibiotic treatment(46). Study reports synergistic interactions of these organism as such that *P. aeruginosa* exoproducts lowers the sensitivity of *S. aureus* biofilm and planktonic populations to vancomycin in CF patients. and also preserves *S. aureus* from other cell wall-active antibiotics and from various classes of protein synthesis inhibitors(47). There seems to be a clear interaction between these organism which are competitive and reciprocally beneficial to each organism in respect to their pathogenicity and colonization(48). Primarily, we assessed the antibiofilm activity of *C. longa* rhizome extracts against *S. aureus* and *P. aeruginosa* and the % inhibition ranged from 26.756-77.72% for two bacterial strains.

The process of primary adhesion between bacteria and abiotic surfaces is generally mediated by nonspecific (e.g., hydrophobic) interactions, whereas adhesion to living or devitalized tissue is accomplished through specific molecular (lectin, ligand, or adhesin) docking mechanisms (49). As a primary fact, Adhesion is the initial step in the complex process of biofilm formation and a prerequisite for colony formation. Thus, targeting this first step would lessen the bacterial cells potential to form biofilms and, ultimately, their virulence. The antibiofilm effects of natural products are majorly seen due to following process, inhibition of formation of polymer matrix, suppression of cell adhesion and attachment, interrupting extracellular matrix (ECM) generation and decreasing virulence factors production, thereby blocking quorum sensing (QS) network and biofilm development. Our test results indicated the anti-biofilm activity of extract by reducing the ability of bacteria to adhere to polystyrene surface in micro titer plate assay as indicated by decrease in absorbance as compared to the untreated cells. These results were also consistent with those found previously(27, 41, 50-52). These results revealed a potential candidate for exploration of new drugs against *S. aureus* and P. aeruginosa biofilm-associated infections

Thus, in this study, the *C. longa* rhizome extract was evaluated for their anti-microbial and anti-biofilm activity against *S. aureus* and *P. aeruginosa* isolates. The ethanolic extract were able to reduce biofilm formation of studied bacterial strains along with promising anti-bacterial activity suggesting the extracts to be as promising candidate for plant derived anti-biofilm compounds. More research is required for the elucidation of the mechanism of action of the anti-biofilm activities of *C. longa* rhizome extract which can be the subject of future studies.

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Authors' contribution

NS, RKS, MP and NS made significant contribution to acquisition of data, analysis, drafting of the manuscript. PP and DPK has made substantial contribution to supervising the research works, conception, design and interpretation of data. RKS and NK participated in research advice, data curation, revising, editing and the manuscript submission. Funding acquisition required was done by G.E.-S.B, M.N, M.A and N.K. Final approval of manuscript was done by all the authors. All authors read and approved the final manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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