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Research paper

Antibacterial activity of Indian propolis and its lead compounds against multi-drug resistant clinical isolates

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ABSTRACT

Introduction: The limited anti-infectious drugs and growing antibiotic resistance among pathogenic bacteria underscore the urgent need to explore novel antimicrobial agents, preferably from a natural source. Propolis is the potent natural antimicrobial agent, which produced by honeybees using various plant exudates. However, it has been minimally studied against multidrug-resistant (MDR) microorganisms. In the present study, the authors have investigated the antibacterial activity of ethanolic extracts of Indian *melifera* propolis (IMP) samples and combinations of their lead compounds against three human clinical isolates.

Methods: Three multidrug-resistant microorganisms (MDRMs) were isolated from a human pus sample, and their molecular identification was carried out by 16S rRNA sequencing. The antibacterial activity of the IMP extracts and different combinations of chrysin, galangin, and phenethyl caffeate was determined by minimum inhibitory concentrations (MIC) using the 96-well plate microdilution method.

Results: Amongst, 19 IMP studied samples, IMP-5, IMP-14, and IMP-16 samples had the most potent antimicrobial activity against three MDR isolates. These samples had antimicrobial efficacy in the order of IMP16 > IMP14 > IMP5. The combinations of chrysin, galangin, and phenethyl caffeate had the lowest MIC values than individual components and above potent IMP extracts.

Conclusions: Certain IMP samples and combinations of chrysin, galangin, and phenethyl caffeate could be the best natural therapeutic agents to control the pathogenicity of MDRMs.

1. Introduction

Antibiotics are one of the most effective antimicrobial drugs of the 20th century, which have saved the lives of millions of people from chronic infections (Dahiya and Purkayastha, 2012; Falagas et al., 2010). However, in the last few decades, some human pathogens have acquired resistance against most known antibiotics (Kumarasamy et al., 2010). The rising rate of resistance among microbes against existing antimicrobial drugs is an alarming threat to global health (Falagas et al., 2010). Considering this, it is becoming pivotal to explore novel antimicrobial agents and therapeutic strategies, which can offer broad-spectrum protection against the multidrug-resistant (MDR) microorganisms and phenotypes (Simoes et al., 2009).

Plants and plant-derived natural products have been used as anti-infectious agents for centuries. Amongst these, propolis is a well-known natural antimicrobial agent produced by honey bees using various plant exudates for their defense from microbes and insects (Kasote, 2017). Propolis has been found to be useful in the treatment of multiple illnesses due to its potent antioxidant, antimicrobial, antiviral, anti-inflammatory, and anticancer properties (Franchin et al., 2018; González-Búrquez et al., 2018; Kasote et al., 2017; Turan et al., 2015). A range of antibacterial and antifungal compounds such as phenols, flavonoids, and terpenoids has been reported to be contained in propolis (De and Drago, 2007). However, the chemical composition of propolis is not uniform across the different types. The flora surrounding beehives is the key determinant of propolis chemical composition (Kasote, 2017).

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Thus, propolis from different geographical and climatic zones can be chemically unique in terms of the composition of bioactives and therapeutic properties.

In a previous study, the authors comparatively studied the chemical profile and antimicrobial activities of Indian *Melifera* propolis (IMP) samples collected from 13 different states of India and demonstrated that ethanolic extract of most of IMP samples were characteristically rich in phenethyl caffeate, and they had a broad spectrum of antimicrobial potential (Kasote et al., 2017). Herein, the antimicrobial activity of 19 different ethanolic extracts of IMP samples have been evaluated against three multidrug-resistant microorganisms (MDRMs) isolated from a human pus sample. In addition, the combinatorial antimicrobial activity of the major bioactive compounds such as chrysin, galangin, and phenethyl caffeate of potent IMP samples have also been assessed against the three MDRMs.

2. Materials and methods

2.1. Isolation and identification of bacterial isolates

The surface area of the infected boil was initially cleaned with sterile distilled water followed by 70 % alcohol to collect a clinical sample. Afterward, the pus specimen was collected by Amies swab (Transwab® Amies Plain Transport) and kept in an incubator for growth in the swab gel medium. After incubation, three morphologically distinct isolates were further separated by streaking on a nutrient agar plate. All isolates were stored at 4 °C until further use.

The cultures of three isolates were further processed for DNA isolation, and their identification was performed by 16S rRNA sequencing analysis (Nandre et al., 2017). The phylogeny tree has been constructed by using MEGA 7.0. Software (Kumar et al., 2016).

2.2. Propolis sample collection and preparation of ethanolic extracts

In this study, 19 different IMP samples were used, which were obtained from 13 different states of India. Details of sample collection have been provided in our previous article (Kasote et al., 2017). The ethanolic extracts of IMP samples were prepared by suspending crude propolis sample in absolute ethanol (1:10, w/v). All extracts were allowed to dry at room temperature and stored at −20 °C until further use (Kasote et al., 2017). For antibacterial activity assay, stock solution of ethanolic extract of each IMP sample was prepared by dissolving it in acetone (16 mg/mL).

2.3. Combinations of chrysin, galangin, and phenethyl caffeate

Chrysin (Alfa Aesar, US), galangin (Alfa Aesar, US) and phenethyl caffeate (TCI, Tokyo, Japan) were procured through local suppliers (Fig. 1). For the antibacterial activity assay, an individual stock solution (16 mg/mL) of chrysin, galangin and phenethyl caffeate was prepared in acetone. Later on, the four different combinations (%, v/v) of chrysin, galangin and phenethyl caffeate were prepared from these stock solutions (as depicted in Table 2). These combinations were prepared based on the antibacterial activity of the individual compounds against MDRM**s** (galangin > chrysin ≥ phenethyl caffeate), and the ratio of relative abundances of these compounds in ethanolic extract of potent IMP samples, IMP-5, IMP-14, and IMP-16 (roughly 11 % phenethyl caffeate, 10 % galangin and 2.5 % chrysin (4.4:4.0:1.0), as reported in the our previous study (Kasote et al., 2017). The intial combination of phenethyl caffeate, galangin and chrysin was in ratio 5.0:4.0:1.0, and the remaining three combinations were established by only changing the ratio of galangin and chrysin, mainly by taking into account the higher antimicrobial activity of the galangin.

2.4. Antibiotic susceptibility testing and determination of minimum inhibitory concentrations (MICs)

All three identified cultures were tested for antibiotic susceptibility by carrying out antibiotic susceptibility testing, and the results were reported as an antibiogram. The cultures were inoculated in nutrient broth and incubated at 37 °C for 24 h on a shaking incubator at 150 rpm. Following overnight incubation, an aliquot (100 µL) of each bacterial suspension (equivalent to 0.5 McFarland standard) was spread on a Mueller Hinton agar plate along with different standard antibiotic discs (Himedia, India) of specific concentrations (Table 1). All the plates were incubated at 37 °C for 24 h, and the zone of inhibition for the standard antibiotic disc was measured. The results of antibiotic susceptibility and resistance were interpreted following Clinical and Laboratory Standards Institute (CLSI) recommended guidelines (Clinical and Laboratory Standards Institute CLSI, 2018).

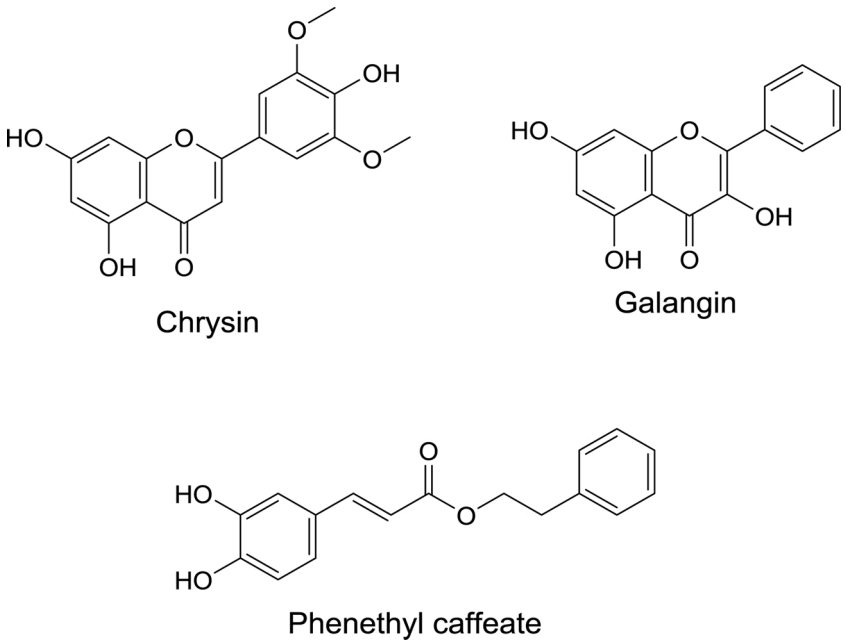


Fig. 1. Structure of major phenolic compounds in the studied Indian *melifera* propolis (IMP) samples.

Table 1
Antibiotic sensitivity of three pathogenic strains isolated from human pus sample.

Sr. No.	Antibiotics	Conc. (μg)	Strain-1 (*Acinetobacter* sp.)		Strain-2 (*Strenotrophomonas* sp.)		Strain-3 (*Enterobacter* sp.)	
			ZoI (mm)	INT	ZoI (mm)	INT	ZoI (mm)	INT
1.	Ticarcillin	75	06	RES	05	RES	18	SUS
2.	Meropenem	10	03	RES	03	RES	NG	SUS
3.	Aztreonam	30	03	RES	NG	SUS	19	SUS
4.	Cefmetazole	30	NZ	RES	07	RES	NG	SUS
5.	Cefotetan	30	NG	SUS	09	RES	11	RES
6.	Chloramphenicol	10	07	RES	NZ	RES	NZ	RES
7.	Cefpodoxime	10	NG	SUS	07	RES	NG	SUS
8.	Amoxycillin	30	09	RES	03	RES	22	SUS
9.	Penicillin-G	10	27	SUS	23	SUS	NG	SUS
10.	Cefonocid	30	05	RES	06	RES	21	SUS
11.	Cefrozil	30	03	RES	06	RES	NG	SUS
12.	Tetracycline	10	NZ	RES	03	RES	NZ	RES
13.	Tazobactam	110	NZ	RES	05	RES	NG	SUS
14.	Rifampicin	2	NZ	RES	NG	SUS	07	RES
15.	Cefixime	5	06	SUS	07	RES	25	SUS
16.	Imipenem	10	04	RES	09	RES	NG	SUS
17.	Ticarcillin/ Clavulanate	75/10	04	RES	03	RES	23	SUS
18.	Cefepime	30	04	RES	04	RES	23	SUS
19.	Actinomycin	10	07	RES	06	RES	03	RES
20	Nitrofurantoin	10	02	RES	08	RES	08	RES

ZoI = Zone of Inhibition; INT = Interpretation; RES = Resistant; SUS = Susceptible; NZ = No zone; NG = No growth.
Interpretation for antibiotic susceptibility and resistance is based on CLSI guidelines 2018; document M100-S23 (M02A11); Performance standards for antimicrobial susceptibility testing.

Table 2
Minimum inhibition concentration (MIC) values of ethanolic extract of Indian *Mellifera* propolis (IMP) samples against three multi-drug resistant microorganisms (MDRMs) isolated from the clinical sample. The (–) indicates the growth of the isolate at the highest concentration of sample i.e., 16 mg/mL. All values are mean of six technical replicates.

Sr. No.	Propolis sample ID	MIC (mg/mL)		
		MDRM-1 (*Acinetobacter* sp.)	MDRM-2 (*Stenotrophomonas* sp.)	MDRM-3 (*Enterobacter* sp.)
1.	IMP-3	–	–	–
2.	IMP-4	–	–	–
3.	IMP-5	2	4	8
4.	IMP-6	<14	–	<14
5.	IMP-7	–	<12	<14
6.	IMP-8	<10	<14	<12
7.	IMP-9	–	–	–
8.	IMP-10	–	–	–
9.	IMP-11	<14	–	<14
10.	IMP-12	–	–	–
11.	IMP-13	<10	<8	<8
12.	IMP-14	1	2	6
13.	IMP-15	<8	<10	<10
14.	IMP-16	1	2	4
15.	IMP-17	<14	<14	–
16.	IMP-18	<12	<10	–
17.	IMP-19	–	–	<10
18.	IMP-20	<14	<12	<14
19.	IMP-21	–	–	–

The minimum inhibitory concentration (MIC) values were determined by a 96 well plate microdilution method (Kasote et al., 2017). The initial inoculums of above MDR clinical isolates- MDRM-1 (*Stenotrophomonas* sp.), MDRM-2 (*Acinetobacter* sp.), and MDRM-3 (*Enterobacter* sp.) were prepared by inoculating respective bacterial colonies in Muller Hinton broth and incubated at 37 °C for 24 h. For MIC determination, an aliquot of 100 μL of media was added to each well-plate followed by 100 μL stock solution (16 mg/mL in acetone) of 19 IMP extracts to first rowwells and serially diluted up to the final well (0.125 mg/mL). For estimating MIC values for pure chrysin, galangin, and phenethyl caffeate, 100 μl of stock solution (16 mg/mL), each compound was serially diluted up to the concentration, 0.125 mg/mL. Similarly, for estimating MIC values for different combinations, the stock solutions (16 mg/mL) of chrysin, galangin and phenethyl caffeate were added in different ratios to make a final volume of 100 μL in the first row and serially diluted up to the concentration, 0.125 mg/mL. MIC values were recorded for IMP samples, pure lead compounds and their combinations after 24 h incubation at 37 °C. The microbial suspension (1×10^8 CFU/mL) of volume 100 μL was added to each well except for sterility control wells. In each assay, negative solvent control, media control, and culture controls were used. Plates were sealed and incubated at 37 °C for 24 h. After incubation, 40 μL of 4 % p-Iodonitrotetrazolium (INT) indicator was added to the incubated plates. The indicator turns pink in the presence of microbial growth. MIC was determined as the lowest concentration of IMP that inhibits the growth of the test microorganisms (no color change). All assays were performed for the average of two biological replicates, each with three technical replicates or six technical replicates, and results were reported as the means of different readings.

3. Results and discussion

3.1. Isolation and identification of bacterial isolates

After the collection and growing of cultures from infected human pus specimens, three different isolates were obtained. All three isolates were identified by partial (800–1300 bp) sequencing of 16S rDNA. These microorganisms belong to gammaproteobacteria, which has a history of opportunistic pathogenesis (Fig. 2). The MDRM-1 showed 96 % identity with *Acinetobacter calcoaceticus*, and it demonstrated a bootstrap of 99 with *A. calcoaceticus* isolate R1. The genus *Acinetobacter* belongs to Gram-negative gammaproteobacteria, and is a major cause of nosocomial infections (Almasaudi, 2018). *Acinetobacter* species found to be resistant to many antibiotics and MDRMs of this genus are causing bacteremia, pneumonia, meningitis, urinary tract infections, and surgical wound infections (Van Looveren et al., 2004). *Acinetobacter baumannii* is an opportunistic pathogen usually seen in immunocompromised patients. *A.calcoaceticus* and *A. baumannii* have close similarities in characteristics and hence alternatively labeled in hospital practices. *A. baumannii* is a known MDR pathogen, which acquires resistance through enzymatic modification of antibiotics, target gene mutation, altered outer membrane permeability, and upregulated multidrug efflux pumps (Ayoub Moubareck and Hammoudi Halat,

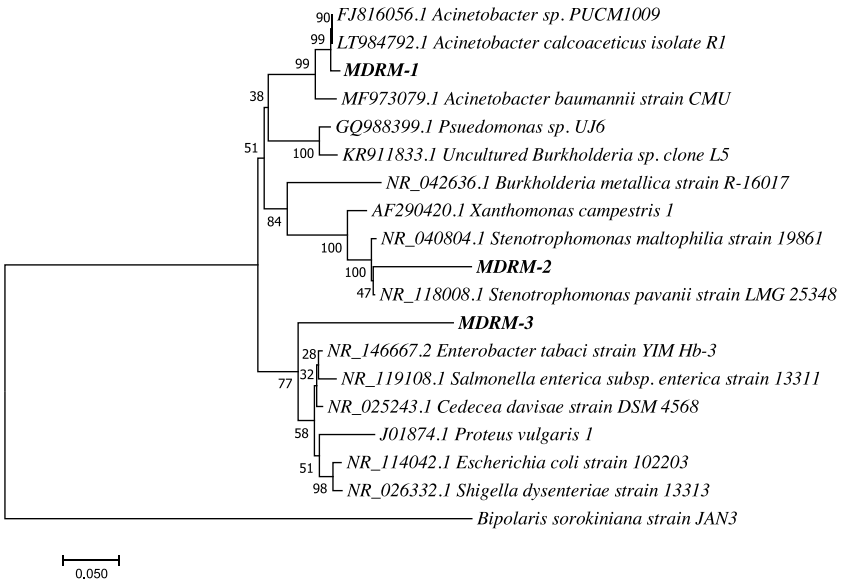


Fig. 2. Neighbour-joining phylogenetic tree of 16S rRNA gene sequence of bacterial isolates from pus sample (bold type) and most closely related species. The optimal tree with the sum of branch length = 1.39478088 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA7.

2020).

MDRM-2 had 92 % similarity with *Stenotrophomonas maltophilia* and *Stenotrophomonas pavanii*. The genus *Stenotrophomonas* also belongs Gram-negative gammaproteobacteria. *S. maltophilia* is also an opportunistic pathogen, usually associated with respiratory tract, soft tissue, bone, blood, eye, heart, and brain infections (Matson et al., 2019). This microorganism was also found to exhibit antibiotic resistance and makes biofilms on epithelial cells. The MDR mechanism of *S. maltophilia* is attributed to genes that encode antibiotic inactivating enzymes, multidrug efflux pumps, and quinolone resistance (Brooke et al., 2017).

MDRM-3 showed 94 % identity with *Enterobacter tabaci* in the phylogenetic tree, whereas the phylogeny showed two different clades (Fig. 2). The phylogeny of these pathogens showed two distinct clades. It showed paraphyletic association with *Salmonella, Cedecea, Proteus, Shigella,* and *Escherichia* genera. These microorganisms belong to gammaproteobacteria, which has history of opportunistic pathogenesis, and the usual sites of their infections are urinary and respiratory tracts. The drug resistance of these microbes is associated with their beta-lactamases and carbapenemase-producing abilities (Bassetti et al., 2016).

3.2. Antibiotic susceptibility testing of bacterial isolates

After identification, an antibiotic sensitivity study of these clinical isolates was performed against standard antibiotics. The antibiogram results for the susceptibility of all three isolates against a panel of antibiotics are represented in Table 1. The results showed that MDRM-1 (*Acinetobacter* sp.) and MDRM-2 (*Stenotrophomonas* sp.) had maximum resistance to different classes of standard antibiotics. In contrast, MDRM-3 (*Enterobacter* sp.) showed a moderate level of resistance towards the selective classes of antibiotics. Despite *Enterobacter* sp. have susceptibility against some of the tested antibiotics, it is still resistant to several commonly used antibiotics. Taken together, all three clinical isolates had MDR traits.

3.3. Antibacterial study of Indian Melifera propolis (IMP) samples

The MIC values were estimated to understand the antibacterial activity of the ethanolic extracts of 19 IMP samples against three MDR clinical isolates. The results of MIC values are shown in Table 2. The majority of IMP samples had moderate antibacterial potential against the tested clinical isolates, except IMP5, IMP14, and IMP16. The observed MIC values of these three IMP samples were in the range of 1−4 mg/mL and had antibacterial efficacy in the order of IMP16 > IMP14 > IMP5. Interestingly, all these samples were highly active against the most noxious pathogen, *Acinetobacter* sp. (MDRM-1), with MIC values of 1−2 mg/mL. On the other hand, these samples moderately inhibited the growth of MDRM-2 (MIC values ranging from 1−4 mg/mL) and had less activity against MDRM-3 (MIC values ranging from 2−4 mg/mL). So far, several studies have demonstrated the antimicrobial and antifungal properties to various propolis samples, including IMP samples against a wide range of microorganisms (Kasote et al., 2017; Tosi et al., 2007). In general, flavonoids, phenolics, terpenes, and terpenoid compounds have been responsible for the antimicrobial activity of propolis (Przybyłek and Karpiński, 2019). Moreover, propolis samples (special propolis extract GH2002 and pacific propolis) were also found to have antimicrobial activity against MDRMs (Astani et al., 2013; Raghukumar et al., 2010). However, little is known about the exact antimicrobial mechanism of propolis samples, and their active principles (Castaldo and Capasso, 2002). Herein, the antimicrobial activity of ethanolic extract of 19 IMP samples has been tested against three MDR clinical isolates. Amongst these, IMP5, IMP14, and IMP16 had potent antibacterial activity. The authors previous study showed that the most potent IMP samples (IMP5, IMP14, and IMP16) were characteristically rich in phenethyl caffeate (11 %), galangin (10 %) and chrysin (2.5 %) (Kasote et al., 2017). On the other hand, the concentration of these compounds was comparatively low in the remaining IMP samples, and most of the samples had only one or two of these compounds. The abundance of these compounds in the three IMP samples can be directly correlated to the observed antimicrobial activity of these samples. The lowest MICs of IMP16 against three tested pathogens can be linked with its high concentrations of chrysin, galangin, and phenethyl caffeate. To validate these findings and understand the synergy among these compounds, the antimicrobial activity of the different combination of chrysin, galangin, and phenethyl caffeate were further investigated.

3.4. Antimicrobial activity of different combinations of chrysin, galangin, and phenethyl caffeate

The results of antimicrobial activities of individual chrysin, galangin, and phenethyl caffeate and their combinations against MDR isolates are shown in Table 3. Individually, chrysin, galangin, and phenethyl caffeate had high MIC values in comparison to their combinations and potent IMP extracts (Table 3). In synergetic activity studies, it was found that the MIC value for MDRM-1 decreased with increasing concentration of galangin and a low amount of chrysin. Galangin exhibited potent

Table 3
Minimum inhibition concentration (MIC) values of four different combinations of lead compounds against three multi-drug resistance microorganisms (MDRMs) isolated from the clinical sample. All values are mean of six technical replicates.

Sr. No.	Compound/ Combination (%, v/v)	MIC (mg/mL)		
		MDRM-1 (*Acinetobacter* sp.)	MDRM-2 (*Stenotrophomonas* sp.)	MDRM-3 (*Enterobacter* sp.)
1.	Chrysin (CH)	8	16	16
2.	Phenethyl caffeate (PC)	16	16	16
3.	Galangin (GL)	4	8	8
4.	PC + GL + CH (50:15:35)	0.5	0.5	2
5.	PC + GL + CH (50:25:25)	0.5	0.5	2
6.	PC + GL + CH (50:30:20)	0.5	1	2
7.	PC + GL + CH (50:40:10)	0.5	0.5	1

activity against all the tested MDRMs, which may be linked with its membrane damage, beta-lactamase and murein hydrolase inhibitory properties (Cushnie and Lamb, 2005; Denny et al., 2002; Ouyang et al., 2018). Both galangin and chrysin are flavonoids, and have close structural similarity but showed distinct biological properties. It has been reported that the amphipathic features of flavonoids play a crucial role in antibacterial activity (Echeverría et al., 2017). Chrysin reported having a bacteriostatic activity against Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa*. However, only a few reports showed the antibacterial property to pure phenethyl caffeate (Meyuhas et al., 2015).

4. Conclusions

Herein, the potent antibacterial activity to certain ethanolic extracts of IMP samples against MDR pathogens, isolated from infected human pus sample has been reported, including the synergistic interactions to their major bioactive compounds, such as chrysin, galangin, and phenethyl caffeate. The overall findings of this study are promising and underline the importance of IMP and its lead components as valuable therapeutic agents to combat MDR pathogenicity. However, this study advocates further in-depth studies on the most potent IMP samples and their bioactives, mainly related to the mechanism of its antibacterial activity and probable *in vivo* toxicity.

CRediT authorship contribution statement

Vinod S.Nandre: Conceptualization, Methodology. **Aditi V. Bagade:** Methodology. **Deepak M. Kasote:** Conceptualization, Original draft preparation, Funding acquisition. **Jisun H. J. Lee:** Software and Editing. **Kisan M. Kodam:** Supervision. **Mohan V. Kulkarni:** Funding acquisition, Resources. **Aijaz Ahmad:** Writing- Reviewing and Editing

Declaration of Competing Interest

The authors report no declarations of interest.

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