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Research Article

Aromatically different Apiaceae spices harbour potential novel antibacterial compounds for drug repurposing against ESKAPE pathogens

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ARTICLEINO **ABSTRACT**

Spices have been utilized over the centuries for culinary and medicinal purposes. Their usage mostly differed as per the different geographical regions and their ethnicity. While they can be of the same class, they might differ in their flavour, aroma, and therapeutic potential. Herein we have explored the widely used south, east, and southeast Asian spices of the Apiaceae family namely cumin (*Cumminum cyminum)* and fennel (*Foeniculum vulgare*) for their therapeutic potential as natural sources of drug repurposing against the infections caused by the multidrug-resistant ESKAPE pathogens.
Essentially, crude extracts of fractions in a series of solvents were prepared and assessed for their preliminary antibacterial potential. All these fractions were analysed through GC-MS and the overlapping constituents were further explored. A stepwise approach of virtual screening followed by pharmacological assessment was taken bioinformatically to reveal the potential top rankers having the druggability against selected virulent proteins from the ESKAPE pathogens. Further molecular dynamics simulation affirmed the finding of top-ranking compounds with their antibacterial potentials. Earlier known as anticancer agents, these compounds hold the promise for drug repurposing from natural sources like spices against the multidrug-resistant ESKAPE pathogens.

Keywords *— Spices, Antibacterial, ESKAPE pathogens, Drug Repurposing, Virulence*

I. INTRODUCTION:

Besides the different parts of herbs, spices find their origin from aromatic lichens, vines, woody shrubs and even trees [1]. Over the years, the usage of spices is ubiquitous and optimum as flavouring and seasoning agents in cuisines [2], with different country expressing their variability in taste [3]. For example, the Indian and Indonesian dishes are often "hot" while the Chinese and Japanese dishes are delicate [4].
Moreover, spices have been used in food preservation and even in medications especially in the south, east and southeast Asian countries. These properties of spices including their specific aroma and unique flavour can be attributed to their constituent phytochemicals or "secondary compounds" as they are secondary to the originating plant's basic metabolism [5]. These chemicals act as protective agents against insects and vertebrates, fungi, pathogens, and parasites [5][6].
Among the spices of southeast Asian cuisines, cumin (*Cumminum cyminum*) and fennel (*Foeniculum vulgare*) are widely used both in the raw and the powder form. Belonging to the Apiaceae family, both cumin and fennel

are characterized by abortifacient, antispasmodic, diuretic, emmenagogic, carminative, and stomachic properties [7]. Cumin contains oleoresin which contributes to the slightly bitter taste while the earthy and smoky note of the aroma is mainly due to cuminol which makes up 2.5 – 4.0% of the seed [8]. Fennel, on the other hand, gives a sweet flavour and a strong note of Licorice and anise seed in aroma coming from the higher percentages of anethole [9]. Owing to their aromatic and carminative properties, both have been used as food flavouring agents and medicinal herbs to treat gastrointestinal illness and upper respiratory tract infections [10].

Besides enhancing food flavour, spices have been utilized for food safety and preservation, which has also been studied in vitro and in vivo [11]. Several compounds isolated from spices have shown good antibacterial activity against different common microorganisms which affects the shelf-life of meat and other food items [12]. For example, the use of cumin and fennel as preservatives in multiple foods that include, meat, fish, dairy products, vegetables, rice and fruit have reported to inhibit the growth of total bacteria by 3.78 log CFU/g when used on meat samples for 15 days at 2°C (Hernández-Ochoa et al., 2014)[13]. Moreover, an in vitro antibacterial study carried out by Cetin et al., (2010) [14], have reported that the essential oil and hexane extracts of fennel exhibited good antibacterial activity against the foodborne pathogens. The main component of both the essential oil and the hexane extract, (E)-anethole, have been reported as the responsible component in inhibiting the bacterial growth. Furthermore, Zellagui et al. (2011)[15] reported a broad range of antimicrobial activity of its acetone extracts against *Enterococcus faecalis, Bacillus subtilis, Escherichia coli, Shigella sp., Streptococcus pyogenes, Staphylococcus aureus, Listeria monocytogenes and Pseudomonas aeruginosa*. Again, good antibacterial activity of the hexane extracts of cumin seeds against *B. subtilis* and *P. aeruginosa* were also reported [16][17]. Therefore, with cumin and fennel inhibiting foodborne pathogens and playing important roles in food preservation, their involvement, in reducing other illnesses caused by multidrug-resistant (MDR) bacterial pathogens, might be imperative [18].

The challenges associated with treating infections caused by the MDR bacteria have been forecasted repeatedly for the last two decades [19][20]. As these MDR pathogens become highly resistant, they caused limitation towards the availability of treatments. Known examples of pathogens that undergo
escalating resistance spreading are the extended spectrum βlactamases, carbapenemases, or the six pathogens summarised by the acronym ESKAPE comprising *Enterococcus faecium, S. aureus, Klebsiella pneumoniae, Acinetobacter baumannii, P. aeruginosa and Enterobacter spp.* [21]. These pathogens have been highlighted by the Infectious Diseases Society of America for being particularly critical in terms of antibiotic resistances [21][22]. They possess several factors like their toxicity and invasiveness which enable them to enhance their virulence [23]. Besides these, mechanisms of virulence and resistance to antimicrobial agents are the necessary characteristics as they enable the pathogenic bacteria to overcome host defence system and survive against antimicrobial therapies [23][24]. Other common virulence and resistance characteristics are direct involvement of cell wall adhesion, efflux pumps or porins and two component systems which activate or suppress the expression of various genes [25]. Thus, alternate therapeutic interventions with unique constituents present in the common spices might necessitate inhibitory studies with such genes or proteins involved in the virulence and resistance mechanisms of these MDR pathogens. The constituents might have been proven effective with some other activities for drug therapies while new antibacterial activities proposed for the same molecules can help in drug repurposing.

In this study, we have carried out a detailed analysis on determining the efficacy of different solvent-based crude extracts of cumin and fennel seeds against the MDR ESKAPE bacterial pathogens. This was followed up by chemical profiling of the extracts through GC-MS to reveal their potential determinants which were further screened, virtually and pharmacologically, to reveal potential novel bioactive compounds. To this end, molecular dynamics simulation was carried out with our shortlisted bioactive compounds, on selected virulent protein targets of the bacteria, and a compound having the highest potential as an effective antibacterial compound from these spices, targeting the ESKAPE pathogens was revealed. We show that certain bioactive compounds, having other therapeutic potentials as anticancer agents, can be repurposed as antibacterial agents, potential enough to address the curing of the infectious diseases caused by the MDR pathogens.

II.MATERIALS AND METHODS

A. Collection of Materials:

Solvents: For crude extractions, HPLC grade organic solvents with increasing polarities were used. These comprised hexane (99% Friendemann Schmidt), ethyl acetate, acetone (99.5% Chemiz, Malaysia) and methanol (99.8%, ChemAR, Systerm, Malaysia). MS grade solvents were used for GC-MS analyses.

Spices: *Cumminum cyminum* and *Foeniculum vulgare* were purchased from spices market. We made it sure that the physical characteristics of the spices remain as close as possible to the description given in scientific literature. The *C. cyminum* seeds are yellow to brownish gray in colour and are elliptical, flat on one side, convex, furrowed, rough on the other, from 5 to 6 mm. in length and about 1.5 mm. in thickness and is elongated in shape with nine protuberances [10]. However, *F. vulgare* seeds are erect and cylindrical, from 4–10 mm in length and bright green in colour.

Bacterial strains: A total of six clinical isolates of ESKAPE pathogens were used in this study, namely, *E. faecium* ATCC®19434™, MRSA MTCC®381128™, *K. pneumoniae* ATCC®700603™, A. baumannii ATCC®19606™, *P. aeruginosa* ATCC®10145™ and *E. aerogenes* ATCC® 13048™.

B. Crude extract preparation:

The crude extracts of *C. cyminum* and *F. vulgare* were prepared by the method mentioned earlier by this group. Briefly, the extraction was carried out directly in four different solvents n-hexane, ethyl acetate, acetone, and methanol, keeping in view their increasing polarity order, nhexane < ethyl acetate < acetone < methanol. Before carrying out extraction, both *C. cyminum* and *F. vulgare* seeds were sequentially washed thoroughly by tap water followed by distilled water to completely remove all the impurities. The washed samples were then left to dry for 1 day. After the seeds were completely dried, they were ground into a fine powdered form with blender. Hereafter, 10 g of powdered samples were dissolved in a 100ml aliquot of the mentioned solvents at a concentration of 1:10 ratio. The extraction was carried out with vigorous shaking for 24 hours with (Yihder LM-530D, Shaker, Taiwan). The resultant solutions were mixed thoroughly by inverting the tube, which was then centrifuged at 4000 rpm for 10 min at 4°C (mEppendorf 5810 R Centrifuge, Germany). The supernatants were subsequently aliquoted into clear glass vials with aluminum screw caps and rubber liners. The extracts were concentrated using rotary evaporator at 4℃ and the dried pellets therefrom, were stored for further use.

C. Preliminary anti-bacterial assays:

Disc diffusion test: The crude extract fractions (CEF) in different solvents, obtained from the spices, were dissolved in 100% dimethyl sulfoxide (DMSO) and were filtered through 0.22 µm syringe filter. For initial screening, each Whatman No.1 filter paper disk (6mm) was loaded with 5µl of the extract at a concentration of 10mg/ml and air-dried for 20 minutes. The testing pathogens were streaked on surface of Mueller-Hinton Agar (MHA) medium (pH 7.3) with a sterile cotton swab. The 6mm filter paper disk was aseptically applied on the surface of the agar plates. Discs with 10% DMSO only were used as negative (solvent) controls while gentamicin (10µg/ml) discs were used as positive controls. The activities of each of the CEF were recorded by measurement of the diameter of inhibition zones. All such experiments were performed with technical triplicates, and thrice, to render three biological duplicates.

Broth microdilution test: Broth dilution procedure was carried out for quantitative measurement of the CEF. This was conducted by dissolving 20 mg of the CEF in 1 ml of DMSO, to yield an initial concentration of 20 mg/ml which was then diluted using double fold serial dilution in a sterile Mueller Hinton broth (MHB). Thereafter, following Clinical & Laboratory Standards Institute (CLSI), each CEF was serially diluted with MHB to obtain final concentrations of 250 µg/mL, 500 µg/mL, 1000 µg/mL, 1500 µg/mL, 2000 µg/mL. On the other hand, the bacterial inoculate were prepared with MHB, incubated at 37℃ for 24 hours and the bacterial concentrations were adjusted to 0.5 McFarland standard. This suspension was diluted to 1:20 to achieve 1 x 105 CFU/ml in phosphate-buffered saline. 5µl of each extract dilution, along with 85µl of MHB and 10µl of bacterial inoculum was released to a well on a 96-well micro-titer plate. A positive growth-control well containing the broth, extract solvent with distilled water and inoculate and a negative control well containing only broth was kept ensuring authenticity of the results. The micro-titer plate was incubated at 37℃ for 16 to 18 hours. The OD reading was analyzed through microplate reader, TECAN. Infinite-M200-PRO.

Statistical analysis: In the present study, all tests were performed in triplicates. Therefrom, the deviation in the data obtained are expressed as the mean ± standard deviation (S.D). The P-values were determined using standard T-test with two-tailed distribution, where * refers to $P \geq 0.05$.

D. Exploratory analyses with GC-MS:

To obtain a complete list of the chemical constituents present in both the spices, their CEF in the volatile solvents like hexane (HX), ethyl acetate (EA), acetone (AC) and methanol (ME), were analysed via gas chromatography followed by mass spectrometric (GC-MS) analysis. The Agilent technologies model 7890B GC System was used for the purpose which was coupled with the Pegasus HT High Throughput TOFMS (Leco Corp., MI, USA). Essentially, the process was initiated with an injection of 1 ml of CEF into the GC-MS apparatus. The components were separated in an inert atmosphere of helium (1.5 mL/min) through Agilent J&W HP-5MS phenyl methyl siloxane analytic column of length 30 m and diameter 0.32 mm having Film of 0.25µm. Other parameters standardized for the method had 80°C initial oven temperature for 2 minutes, gradually raised at the rate of 3°C/min to 300°C followed by 5 minutes solvent delay and temperatures of 225°C inlet line temperature with 250°C ion-source temperature. The run time was kept for 64 minutes and the readings for mass spectra were recorded at 70 eV having acquisition mode-scan of 20-1000 amu. Finally, the spectral data were explored with libraries of NIST database to decipher and document the phytochemicals present. Therefrom, selected compounds were used to confirm their ability to inhibit the antibacterial activity of selected MDR pathogens, explained in the forthcoming sections.

E. In silico analysis of chemical constituents:

Target model acquisition: To estimate the antibacterial potential of the chemical constituents obtained from GC-MS analysis, these were subjected to in-silico analyses against selected target proteins of the six

ESKAPE pathogens. The target proteins from each bacteria were referred from the virulence factors (VFs) of the corresponding bacterial proteome listed in the Virulence Factor Database (VFDB). Thereafter, they were selected from the scientific literature based on their functional relatedness to MDR in the respective bacteria. The proteins are LiaR for *E. faecium*, Tst for MRSA, Cdd for *K. pneumoniae*, OmpA for *A. baumannii*, PcrH for *P. aeruginosa* and OmpK35 for *E. aerogenes*. Their full-length X-ray crystallographic three-dimensional structures were retrieved from the Protein Data Bank (PDB) (www.pdb.com) focussing on good resolution. These are 5HEV for *E. faecium*, 3TSS for MRSA, 6K63 for *K. pneumoniae*, 4G4Y for *A. baumannii*, 2XCC for *P. aeruginosa* and 5O78 for *E. aerogenes*, for the respective proteins mentioned above here

Ligand molecule preparation: Following the selection of the target proteins, the 3D structure of the chemical constituents of *C. cyminum* and *F. vulgare*, revealed through chromatographic analysis, were obtained. To be used as ligands, the SDF files of all these compounds were downloaded from PubChem database
(https://pubchem.ncbi.nlm.nih.gov/). These SDF files were converted into PDBQT files by utilizing OpenBabel [26] integrated into the PyRx software. In addition, MMFF94 force field was set for ligand optimization followed by energy minimization for 1000 steps by the conjugate gradient algorithm [27].
Receptor protein preparation: The X-ray crystallographic structures, retrieved from PDB, were used as receptor in the molecular docking simulation [28]. The macromolecule receptors were prepared utilizing AutoDockTools 4.2 [29]. Prior to PDBQT file generation of the retrieved PDB structures, non-amino acid residues were discarded followed by structural optimization wherein polar hydrogen and Kollman charges were added. The grid of each macromolecule receptor was set to encompass the predicted docking sites [29].
Druggable pocket prediction: For structure-based molecular docking, druggable pockets of each of the selected virulence protein targets were identified using P2RANK from PrankWEB server (https://prankweb.cz/) [30]. This computational tool predicts the druggable pockets and generates scores from protein structures by utilizing templateindependent machine learning approach. The top-ranked predicted pockets were selected and used in molecular docking analysis.
Virtual ligand screening: Molecular docking was performed for virtually screening the compounds obtained from chromatographic analysis based on the receptor proteinligand binding affinity threshold score. This was executed via AutoDockVina tool which predicts the magnitude of ligandprotein interaction in its scoring function (binding affinity) [31][32]. Docking grids of all macromolecule receptors, were adjusted into squares of 30 Å with x, y, and z coordinates to define the binding sites (Table S10). The better binding energies (kcal/mol) of docked ligand-protein complexes were selected for further analysis. The interaction of the ligandprotein complexes was visualized using BIOVIA Discovery Studio Visualizer version 20.1.0 tool [33].
Pharmacological properties evaluation: To enhance the reliability in filtering the *C. cyminum* and *F. vulgare* bioactive compounds, from the virtual ligand screening analysis, the most prominent chemical components were shortlisted further for their pharmacological properties. This included the properties of ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicity), oral bioavailbility (OB), and molecular information for each chemical compound which were evaluated using SwissADME server (http://www.swissadme.ch/) [34].
Molecular dynamics simulation: To understand the dynamic behaviour and the ligand-bound protein interaction pattern, GROMACS simulation tool was used [35]. This tool was accessed through a publicly available server, WebGro (www.simlab.uams.edu). The ligand-bound structures were prepared using GROMOS96 43a1 force field solvated by the Single point charge (SPC) water model and were fixed in a periodic cubic solvated box. The charged ligand topology file was externally prepared through the PRODRG server (http://prodrg1.dyndns.org/) [36].

III. RESULTS:

A. Antibacterial activity of spices crude extracts by disc diffusion technique:

An initial screening of the antibacterial activity of the different solvent based CEF of cumin and fennel seeds were carried out in biological and technical triplicates. In each case, however, the solvent control (SC), DMSO, did not show any bacterial inhibition and thus, was not included in Table 1. Based on Table 1A, EA and AC CEF of *C. cyminum* showed zone of inhibition against *K. pneumoniae* and *P. aeruginosa*. According to Table 1B, EA, AC and ME CEF of *F. vulgare* showed medium zone of inhibition against MRSA and *E. aerogenes*. As per the results obtained, even though the zone of inhibition produced by the some of the CEF were moderate, the results were unclear with respect to all the bacteria which made it impossible to make an inference. Therefore, broth dilution test was also carried out to obtain a clear picture of their activities.

B. Antibacterial screening of crude extracts via broth microdilution assay:

Broth microdilution assay was carried out by determining the percentage inhibitory activity of CEF of both the spices against the tested ESKAPE pathogens. The results obtained for HX and AC CEF of *C. cyminum* showed lowest MIC against MRSA (Figure 1b) and *P. aeruginosa* (Figure 1e) whereas, EA and AC of *F. vulgare* CEF showed lowest MIC against *E. faecium* (Figure 1g) and *A. baumannii* (Figure 1j). Among the CEF of *C. cyminum*, all the fractions showed good inhibitory activity against all the tested ESKAPE pathogens within the

range of 1500µg/ml to 2000µg/ml concentration (Table S1). The same observation is applicable for *F. vulgare*, within the range of 1000µg/ml to 1500µg/ml (Table S2). The results obtained from the EA and AC CEF of *F. vulgare* and *C. cyminum* showed prominent inhibitory activity compared to the other extracts (Figure 1). Based on the results obtained, however, all the CEF showed prominent antibacterial efficacy, except for *F. vulgare* ME extract. This prompted us to use these CEF to further process them for the identification of bioactive compounds through GC-MS analyses.

C. Identification of volatile constituents by Gas Chromatography – Mass Spectrometry

GCMS analysis was carried out for all four CEF of *C. cyminum* and 3 fractions of *F. vulgare* (HX, EA and AC) owing to their promising antibacterial activities. The analysis of *C. cyminum* CEF revealed, 109 bioactive compounds from HX, 129 from EA, 236 from AC and 230 from ME (Table S3 - S6). The analysis of *F. vulgare* CEF revealed, 118 bioactive compounds from HX, 144 from EA and 214 from AC (Table S7 - S9). Based on the analysis, we found that there was presence of similar chemical compounds repetitively in all the CEF analysed and, these repetitive chemical compounds were also found abundantly (Table S1-S9). Therefore, the repetitive compounds present in all the solvents were filtered out for further downstream analysis. We filtered out 26 such compounds from *F. vulgare* (Table 2) and 13 from *C. cyminum* (Table 3). Among these compounds, only 8 for fennel and 4 for cumin, have been earlier reported with antibacterial activities.

D. Shortlisting of chemical compounds by virtual and pharmacological screening:

Following the GC-MS analysis, the common chemical compounds obtained from the different CEF of the spices were further analysed through virtual screening. The virtual screening was conducted on all the 13 chemical compounds from *C. cyminum* and 26 from *F. vulgare* upon a set of 6 different key proteins (Table 4) into their top ranked druggable pockets predicted by "P2rank" (Table 5). These proteins are LiaR for *E. faecium*, Tsst for MRSA, Cdd for *K. pneumonia*, OmpA for *A. baumannii*, PcrH for *P. aeruginosa* and Omp35 for *E. aerogenes*. These proteins, earlier reported to be involved in the MDR of the ESKAPE pathogens, were then used to analyse in determining and proving the antibacterial activity of the prominent chemical constituents of *C. cyminum* and *F. vulgare*.

The docking based virtual screening (Table S10) of the common chemical constituents or ligands shown in Tables 2 and 3 with the selected proteins of Table 4 helped us analyse the most prominent ligands which were then use for further studying their pharmacological properties. The potential druggable ligands were chosen based upon the binding energies of the ligands with proteins which are around and lower than -7 kcal/mol (Table S11-S12, Figure 2). Thereafter, the potential drug candidates were identified based on their predicted pharmacological properties. For this, following good binding energies, good gastrointestinal (GI) absorption, bad BBB permeability, and non-P-glycoprotein (PGP) substrates for absorption properties were considered along with non-cytochrome P450 inhibitors for metabolism. Moreover, violations of the drug-likeness rules were avoided by following five druggability rules, namely, the Lipinski, Ghose, Veber, Egan, and Muegge rules [37][38]. Lastly, higher Abbot Bioavailability Score was utilized to predict the probabilities of drug bioavailability to be more than 10% upon oral intake [37][38].

Based on the structure-based virtual screening and the pharmacological properties of each of the chemical compounds, Phenol, 2,4-bis(1,1-dimethylethyl)- (coined BisP) from both *C. cyminum* and *F. vulgare* was found to be one of the most potential druggable molecules. This is due to its potential druggability to 4 proteins, namely, Tsst, Cdd, PcrH and Omp35 with stable binding energy lower than -6.6 to -8.2 kcal/mol. Besides that, it also had good pharmacological properties. There was, however, a problem with violating the BBB permeability and being inhibitory to one of the cytochromeP450 enzymes (Table S11-S12). On the contrary, a derivative of isoquinolinedione namely, 1,8(2H,5H)-Isoquinolinedione, 6,7-dihydro-3-hydroxy- (coined ISQ) from cumin, exhibited druggability almost quite acceptably with 1 Muegge rule violation. Like BisP, this also showed acceptable binding energies with the proteins, leaving out LiaR and Omp38. An even better compound from fennel, namely, 2H,6H-Benzo[1,2-b:5,4-b']dipyran-2,6dione, 7,8-dihydro-8,8-dimethyl- (coined 7BD) stands out as the best of the lot, obeying all the pharmacological properties (Table S12). Binding energies against LiaR and Omp38, however, are not promising, just like the other ligands mentioned above here.

E. Validating inhibitory effects of selected compounds by Molecular Dynamics Simulation:

Molecular Dynamics simulations (MDS) were carried out for 50 nanoseconds for ISQ, BisP and 7BD LigandsTsT/LiaR/Cdd complexes for MRSA, *E. faecium* and *K. pneumoniae*, respectively, to observe the stability of the interactions between the chemical ligand and the protein receptors. Throughout MD simulations, the ligands showed some variability in the retainment in the docking pocket of respective protein receptors, with *K. pneumoniae* Cdd showing the best binding for all the three ligands while *E. faecium* LiaR showing the least wherein all the ligands seemed to be escaping from their initial binding pockets (Figure 2). This was captured through their binding energies as well, being around -8 Kcal/mole for *K. pneumoniae* while these were around -5 and around -7 for *E. faecium* and MRSA, respectively. Besides, the total number of receptorligand intermolecular hydrogen bonds were maintained stably between 1 and 2 in all the complexes, with intermittent fluctuations (Figure 3, 4). We also observed stable active residues in MRSA Tst-ISQ and MRSA Tst-7BD (SER 15, LYS 67, ARG 68), MRSA Tst-BisP (LYS 67), and *E. faecium* LiaR-ISQ and *E. faecium* LiaR-7BD (LYS 67) complexes, as well as in *K. pneumoniae* Cdd-ISQ (PHE 233) and *K.*

pneumoniae Cdd-7BD (ALA 103) complexes (Figure 2).
In all MD simulation systems, the root-mean-square fluctuations of the ligand ISQ complexed with *K. pneumoniae*-Cdd and MRSA-Tst receptors were maintained at around 0.5nm (5Å) (Figure 3). Again, the RMSD of BisP and 7BD were maintained within 0.5nm mostly in the *K. pneumoniae*-Cdd, MRSA-Tst and *E. faecium*-LiaR receptors (Figure 4). The fluctuations in the ligand RMS were relatively higher in *E. faecium* (around 0.5 nm), compared to those in *K. pneumoniae* and MRSA (Figure 3 and 4).

IV. DISCUSSION:

An emerging increase in MDR among ESKAPE pathogens have raised concerns for alternative antimicrobial drugs to combat these pathogens in future. However, many studies are being carried out on developing different types of antimicrobial therapy with the most prominent ones being antibiotics in combination, bacteriophage therapy, antimicrobial peptides, and silver nanoparticles. Besides these, huge research is also being done on plants. Based on the varieties of studies, natural products, widely derived from spices, have been exploited as a resource in drug development and repurposing. This is because, spices were utilized as traditional medicines in poor or developing countries since ancient times as it has been the key source for drugs and alternative medicines to fight against infectious diseases [39]. Spices have been known to possess good antibacterial properties. Besides adding them into our daily cooking which provides us good nutrition, spices might have many other potentials that are yet to be discovered.
For the development of antibacterial agents, novel biologically active compounds can be explored for their diverse functions. Small organic molecules are always studied in pharmaceutical and biochemical fields, as they have the potential to provide powerful effects on macromolecules and can serve as hits against different biological targets while being able to be used in biological screening (Ji, Li, & Zhang, 2009). In this study, we carried out detailed observation on the two aromatically different Apiaceae spices C. cyminum and F. vulgare which are largely found in southeast Asian countries. Both C. cyminum and F. vulgare were extracted into four different solvents namely, hexane (HX), ethyl acetate (EA), acetone (AC) and methanol (ME) to yield potential crude extract (CEF) fractions. These CEF were then tested against the six ESKAPE pathogens namely, E. faecium, MRSA, K. pneumoniae, A. baumannii, P. aeruginosa and E. aerogenes. Since both spices showed good inhibitory activities, a GC-MS analysis was carried out for all the CEF except for the ME CEF of F. vulgare as it showed low inhibitory activity compared to the other extracts. Despite both the spices belonging to the Apiaceae family, only 26 and 13 chemical compounds, for fennel and cumin, respectively, were found to be overlapping across all their individually chromatographed CEF. A molecular docking based virtual screening, for all these overlapping compounds, were carried out, followed by analyzing their pharmacological properties against the selected proteins involved in the resistance mechanism of the ESKAPE bacterial strains. Further molecular dynamics simulation study finally portrayed compound(s) which could be potential drug candidate for future pharmacological industries.
Both C. cyminum and F. vulgare were extracted with four different solvents of increasing polarity. This was done to understand the potential of the solvents in extracting the essential chemicals contributing to the biological activities exhibited by both the spices. Preliminary antibacterial assays were carried out for all the CEF against the selected ESKAPE bacterial strains. Based on the results obtained, almost all the CEF showed good inhibitory effect except for ME CEF of F. vulgare which responded moderately compared to the other CEF. A moderate zone of inhibition was observed in the AC and EA C. cyminum CEF and in EA, AC and ME of F. vulgare CEF. Since the results obtained through zone of inhibition were moderate and unclear, a minimum inhibitory concentration (MIC) was carried out to obtain a better understanding of the potential activities of the CEF. For C. cyminum, the lowest MIC value within the range of 1500µg/ml to 2000µg/ml (Table S1) was observed for the CEF among which AC showed the best inhibitory effect followed by HX, EA and ME against all the tested ESKAPE pathogens. These CEF were effective against E. faecium and A. baumannii compared to other strains which had moderate activity. For F. vulgare, the lowest MIC value was observed within the range of 1000µg/ml to 1500µg/ml (Table S2) for the CEF with similar results of AC being the most promising crude extract followed by HX, EA, and ME. F. vulgare also showed the best inhibitory effect towards E. faecium and A. baumannii at the same concentration.
As per our findings, CEF of AC and HX for C. cyminum and AC for F. vulgare showed more promising inhibitory activity compared to the other CEF even though they showed good inhibitory activities. This was quite in line with the study reported by Persaud et al. (2019) [16] which showed HX CEF of C. cyminum to exhibit good antibacterial activity against B. subtilis and P. aeruginosa. Besides that, a study carried out by Agarwal et al., (2019) [17] also indicated that HX CEF of cumin seeds to possess potential antibacterial activity. In this case, however, different cumin genotypes were used to evaluate the antibacterial as well as their metabolites contents. For example, minimum inhibition (58 %) was exhibited in S. aureus with GC-1 and RZ-341 varieties whereas it was 62 % with GC-4 and RZ-209 and the maximum inhibition (66 %) was recorded with RZ-19 (Agarwal et al., 2019[17]). Again for F. vulgare, Zellagui et al., (2011) [15] reported good antimicrobial activity of especially AC CEF against E. faecalis, B. subtilis, E. coli, Shigella sp., S. pyogenes, S. aureus, L. monocytogenes, P. aeruginosa and S. cerevisiae. In fact, the screened antibacterial activities of these CEF showed promising antibacterial activities against all the tested pathogens. Thus, the earlier reported studies resonate with our findings. Therefore, for our case, a GC-MS analysis was carried out for all C. cyminum CEF

except for F. vulgare, in which HX, EA and AC CEF were only further chromatographically analysed because ME CEF showed less inhibitory activity compared to the other fractions.

Our gas chromatographic-mass spectroemtric analysis showed vast number of bioactive compounds present in both C. cyminum (Figure S1 – S4) and F. vulgare (Figure S5 – S7). Since all C. cyminum CEF and HX, EA, and AC of F. vulgare showed good inhibitory activities, we expected these crude extracts to harbour overlapping important bioactive compounds (Table S3-S6 and Table S7-S9). Thus, we have found 13 and 26 chemical compounds for cumin and fennel, overlapping across all the CEF analysed via GC-MS. Among these analysed compounds from C. cyminum, only 4 out of 13 compounds were reported to have antibacterial activities (Table 3). For example, the antibacterial effects of (-)Carvone were reported by (Porfirio et al., 2017) [40], àPhellandrene were reported by (Iscan et al., 2012) [41], pCymene were reported by (Gomori et al., 2018) [42] and Phenol, 2,4-bis(1,1-dimethylethyl)- were reported by (Padmavathi et al., 2015) [43]. Again, for 26 overlapping compounds from the three different analysed CEF of F. vulgare, only 7 compounds were reported to have antibacterial properties (Table 2). For example, the antibacterial activity of estragole were reported by da Costa et al. (2021), anethole by Kwiatkowski et al. (2019) [44], carveol by Guimarães et al. (2019) [45], oleic acid by Le et al. (2010), benzaldehyde, 4-methoxy- by Mbah et al. (2017) [46] and Phenol, 2,4-bis(1,1-dimethylethyl)- by Padmavathi et al. (2015) [43]. After carrying out this screening analysis, we found many unreported compounds as well as the specific compounds antibacterial activity has not been analysed yet. As of now, based on the reported compounds stated above, Phenol, 2,4-bis(1,1-dimethylethyl)- was not reported as a compound found in *C. cyminum* and *F. vulgare*. Our findings revealed, for the first time, the presence of this compound in both *C. cyminum* and *F. vulgare.*

To further screen down the prominent compounds for future usage, a virtual screening study was carried out which has been known to be an effective way to select out prominent compounds through hit identification and lead optimization [37][38][47]. A similar approach of drug lead discovery through virtual screening analysis was carried out in our studies on *C. cyminum* and *F. vulgare*. With a total compound of 39 from both *C. cyminum* and *F. vulgare,* a computational analysis was carried out. This entailed selection of a group of target virulent proteins from the MDR ESKAPE pathogens followed by the virtual screening with those 39 compounds on to the targets through molecular docking and finally analysing their bioavailability and toxicity through pharmacological analysis. These analyses particularly revealed the best of the bioactive compounds from both *C. cyminum* and *F. vulgare* to be Benzo[1,2-b:5,4-b']dipyran2,6-dione,7,8-dihydro-8,8-dimethyl- , 1,8(2H,5H)Isoquinolinedione,6,7-dihydro-3-hydroxy- and Phenol,2,4bis(1,1-dimethylethyl)- (Table S11-S12). These were coined by us as 7BD, ISQ and BisP for the ease of use. While 7BD and ISQ are newly reported compounds without any known activities, BisP is also called as 2,4-di-tert-butylphenol (DTBP) and has the role of bacterial metabolite, an antioxidant, and a marine metabolite. It is an alkylbenzene and a member of phenols (National Center for Biotechnology Information, 2022). It has been widely used as an antioxidant light protection agent, UV stabilizers and acts as a chemical intermediate [48]. There are several studies which showed that this chemical compound has promising drug properties. This chemical compound has an important biofilm inhibition property. Studies were reported in which DTBP extracted from seaweed Gracilaria gracilis has exhibited a dose dependent antibiofilm activity as well as anti-quorum sensing [49][50]. Our study is the first to reveal that *C. cyminum and F. vulgare* also contains the compound DTBP, thereby proving the antibacterial potential of these spices, albeit partially.

The three lead compounds 7BD, ISQ and BisP were then used for MDS to further affirm their stability for antibacterial potential upon complexing with the virulence proteins that were selected to examine in this study. The development of antibiotic resistance has become ever increasing concern in this era. One of the factors which influences such antibiotic resistance is enhanced virulence in these pathogens [51]. Therefore, different types of virulent proteins were selected from different bacteria. A total of 6 different key virulent proteins were selected accordingly. These are LiaR for *E. faecium*, Tst for MRSA, Cdd for *K. pneumoniae*, OmpA for *A. baumannii*, PcrH for *P. aeruginosa* and Omp35 for *E. aerogenes*. Among these, LiaR plays an important role in the three-component LiaFSR signaling pathway for *E. faecium* and alters its membrane homeostasis resulting in resistance to DAP (daptomycin) and antimicrobial peptides [52]. TSST-1, encoded by the gene tst is reported as one of the most important virulence factors of MRSA [53]. The protein Cdd of *K. pneumoniae* codes for Cytidine deaminase which scavenges exogenous and endogenous cytidine and 2'deoxycytidine for UMP synthesis [54]. Again, Omp38 of *A. baumannii* induces apoptosis in human cell lines through caspase-dependent and AIF-dependent pathways [55]. It has been reported that purified Omp38 enters host cell and localizes to the mitochondria, which presumably leads to a release of proapoptotic molecules such as cytochrome c and AIF (apoptosis-inducing factor) [55]. PcrH of *P. aeruginosa* plays an important role in chaperone-mediated protein complex assembly, and protein secretion by the type III secretion system [56]. The protein Omp35 of *E. aerogenes* acts as porin for the ion transmembrane transport.

Additionally, upon screening of the receptor-ligand complexes, the binding energies were found to be the best for Cdd for *K. pneumoniae* (around -8Kcal/mol) followed by Tst for MRSA in the mid-range of -7Kcal/mol and LiaR for *E. faecium* in the low range of around -4 Kcal/mol. Thus, these were chosen for further MDS studies based on their representative range of binding energies. The detailed MDS studies led to the affirmation of the complexes of Cdd for *K. pneumoniae* followed by Tst for MRSA and LiaR for *E. faecium*. Therein, the ligands 7BD turn out to be the best followed by ISQ and BisP. Among these, BisP has already been reported, recently, with some antibacterial activities. 7BD is also known as Graveolone and is a coumarin class of compound which

have been reported to be used as anticancer agents [57]. ISQ belongs to the class of isoquinolinedione which have been proposed as anticancer agents [58][59]. With a different backdrop of the therapeutic potentials of 7BD and ISQ, it becomes intriguing to explore their potential as antibacterial agents. It definitely needs detailed studies to prove this new potential, but days might not be far for the future researchers to rely on such natural product-based drug repurposing agents for the treatment of bacterial infections, especially, the MDR ESKAPE pathogens.

V. TABLES:

Table 1| Antibacterial activity of A) *C. cyminum* and B) *F. vulgare* crude extract fractions.

A) Inhibition zone diameter (mm)

Extracts	*E. faecium* ATCC 19434	MR *S. aureus* MTCC 381123	*K. pneumoniae* ATCC 700603	*A. baumannii* ATCC 19606	*P. aeruginosa* ATCC 10145	*E. aerogenes* ATCC 13048
HX	-	-	-	-	-	-
EA	-	-	14.7 ± 2.31	-	-	-
AC	-	-	-	-	-	-
ME	-	-	-	-	11.3 ± 1.15	-

B) Inhibition zone diameter (mm)

Extracts	*E. faecium* ATCC 19434	MR *S. aureus* MTCC 381123	*K. pneumoniae* ATCC 700603	*A. baumannii* ATCC 19606	*P. aeruginosa* ATCC 10145	*E. aerogenes* ATCC 13048
HX	-	-	-	-	-	-
EA	-	9.33 ± 1.15	-	-	-	10.7 ± 1.15
AC	-	10.7 ± 1.15	-	-	-	-
ME	-	12.7 ± 1.15	-	-	-	-

"-" = No activity, HX = Hexane, EA = Ethyl acetate, AC = Acetone, ME = Methanol. The data are from an average of three independent experiments performed in technical triplicates & expressed as mean ±standard error

Table 2 | Compounds present in all the solvent CEF of *F. vulgare*:

NO	Identified compounds	Molecular Formula	RT (min)	Area %	Antibacterial activity report
1.	Estragole	$C_{10}H_{12}O$	411.67	53526196	Reported
2.	Anethole	$C_{10}H_{12}O$	533.713	8.97E+08	Reported
3.	2-Propanone, 1-(4-methoxyphenyl)-	$C_{10}H_{12}O_2$	672.223	13263675	Not reported
4.	10-Nonadecanone	$C_{19}H_{38}O$	2731.69	42487947	Not reported
5.	2,6-Dimethyl-1,3,5,7-octatetraene, E,E-	$C_{10}H_{14}$	313.132	1185266	Not reported
6.	Benzenemethanol, à-ethyl-4-methoxy-	$C_{10}H_{14}O_2$	1101.33	2606777	Not reported
7.	Carveol	$C_{10}H_{16}O$	455.261	1273717	Reported
			1636.62	2.33E+08	
9.	1-Cyclohexyldimethylsilyloxybutane	$C_{12}H_{26}OSi$	2118.95	93373	Not reported
10.	2H,6H-Benzo[1,2-b:5,4-b']dipyran-2,6-dione, 7,8dihydro-¹,8-dimethyl-	C14H12O5	1657.33	293533	Not reported
11.	4-Methoxycinnamaldehyde	$C_{10}H_{10}O_2$	928.361	380657	Not reported
12.	Benzaldehyde, 4-(1-methylethyl)-	$C_{10}H_{12}O$	467.545	2035439	Not reported
13.	Benzaldehyde, 4-methoxy-	$C_8H_8O_2$	486.137	83391296	Reported
14.	Benzene, (1,3-dimethoxypropyl)-	$C_{11}H_{16}O_2$	1988.01	276114	Not reported
15.	Bicyclo[2.2.1]heptan-2-one, 1,3,3-trimethyl-	$C_{10}H_{16}O$	277.807	65933716	Not reported
16.	Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-, (1S)-	C10H16O	343.145	1526319	Not reported
17.	Bicyclo[2.2.1]heptane, 2,2-dimethyl-3-methylene-, (1R)- (Champene)	C10H16	460.374	67055	Not reported
[1] 8.	Oleic Acid	$C_{18}H_{34}O_2$			Reported

18.	Bicyclo[3.1.1]hept-2-ene-2-methanol, 6,6-dimethyl- (Myrtenol)	C10H16O	334.347	13833	Not reproted
19.	Carbamic acid, N,N-diisopropyl-, 3-(4methoxyphenyl)propyl ester	C17H27NO3	2165.06	121554 5	Not reported
20.	(-)-Carvone	$C_{10}H_{14}O$			Reported
21.	Cyclohexene, 1-methyl-4-(1-methylethenyl)-, (S)- (Limonene)	$C_{10}H_{16}$			Not reported
22.	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	1430.35	5660612	
			472.99	4905937	
			218.578	14030321	
			539.39	8573832	
					Not reported
23.	p-Cymen-7-ol	$C_{10}H_{14}O$			Not reported
24.	Phenol, 2,4-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	858.741	2210330	Reported
25.	Phenol, 2-methoxy-3-(2-propenyl)-	C10H12O2	635.106	806595	Reported
26.	Phenol, 2-methyl-5-(1-methylethyl)- (Carvacrol)	$C_{10}H_{14}O$	556.388	500075	Not reported

Table 3 | Compounds present in all the solvent CEF of *C. cyminum*

No	Identified compounds	Molecular formula	RT (min)	Area %	Antibacterial activity
1.	(-)-Carvone	C10H14O	748.849	1639031	Reported
2.	1,3,8-p-Menthatriene	C10H14	593.971	18268809	Not reported
3.	1,3-Benzodioxole, 4-methoxy-6-(2-propenyl)-	C11H12O3	868.402	1582637	Not reported
4.	1,8(2H,5H)-Isoquinolinedione, 6,7-dihydro-3hydroxy-	C9H9NO3	1182.37	600163	Not reported
5.	12-O-Acetylingol 8-tiglate	C27H38O8	1773.2	1.38E+08	Not reported
6.	2-Caren-10-al	C10H14O	529.463	16259428	Not reported
7.	4-Carene, (1S,3R,6R)-(-)-	C10H16	255.132	1507239	Not reported
8.	à-Phellandrene	C10H16	198.293	58866	Reported
9.	Apiol	C12H14O4	1009	60513661	Not reported
10.	Benzaldehyde, 4-(1-methylethyl)-	C10H12O	468.176	4.01E+08	Not reported
11.	Benzene, 1-methyl-3-(1-methylethyl)-	C10H14	214.628	214.628	Not reported
12.	p-Cymene	C10H14	685.702	532360	Reported
13.	Phenol, 2,4-bis(1,1-dimethylethyl)-	C14H22O	858.542	748314	Reported

Table 4 | Selected proteins data used in virtual and pharmacological screening:

Bacteria Species	Uniprot ID	Gene Name	Role	Length	PDB ID	CHAIN ID	Resolution
E. faecium	A0A6N3FD85	*liaR*	Three-component LiaFSR signaling pathway	210	5HEV	A,B,C	3.19
MRSA	P06886	*tst*	Toxic shock syndrome toxin	234	3TSS	A	1.9
K. pneumoniae	A6TBN1	*cdd*	Cytidine deaminase (zinc-dependent enzymes)	294	6K63	A,B,C,D	2.07
A. baumannii	Q6RYW5	*omp38*	Outer membrane protein	356	4G4Y	A,B,C,D, E,F,G,H	1.7
P. aeruginosa	Q9I325	*pcrH*	Regulatory protein PcrH (chaperone)	167	2XCC	A,B	2.13

E. aerogenes	A0A0H3FYJ7	*omp35 (EAE_15245)*	Outer membrane porin protein	359	5O78	A,B,C	2.85

Table 5 | The P2 Rank Predicted Druggable Pockets

Species	Bacterial name	Protein PDB ID	PDB Structure CHAIN ID	PDB Structure Pocket Residues	P2Rank
E. faecium	LiaR	5HEV	A,B,C	284 285 286 287 309 311 313 320 471 472 528 552 553 560 561	
MRSA	Tst	6K63	A,B,C,D	93 97 98 100 103 105 119 870 871 891 898 899 1008 1008 1008 1013 1024 1026 1027 1029 1031 1055 1056 1057 1058 1059 1060 1061 1062 1065 1069	
K. pneumoniae	Cdd	4G4Y	A,B,C,D,E,F,G,H	501 502 517 518 519 521 536 537 538 653 656 670 671 673 712 713 715 718 720 722 725 739 740 741 754 755 756 757 758 759 771 772 924 938 939 940 942 950 961 962 965 966 971 975 976 977 979 980 1779 1780	
A. baumannii	Omp38	2XCC	A,B	374 375 393 407 408 754 759 773 884 885 898 899 902 904 916	
P. aeruginosa	PcrH	5O78	A,B,C	135 137 162 164 166 191 193 194 204 411 413 445 448 449 451 467 470 494 495 496 502 690 692 730 732 733 751 752	
E. aerogenes	Omp35 (EAE_15245)	3TSS	A	79 80 81 95 96 108 109 213 214 228 229 231 233 234 249 409 428 429 430 445 519 521 523 532 536 537 539 684 690 693 694 695 696 722 723 724 725 733 752 754 755 772 773 774 775 776 780 783 784 788 796 803 808 826 830 859 860 861 882 883 927 937 938 999 1906 1977 1978 1979 2236 2237 2238 2239 2240 2262 2263 2264 2316 2317 2334 2335 2555 2556 2576 2577	

VI. FIGURES:

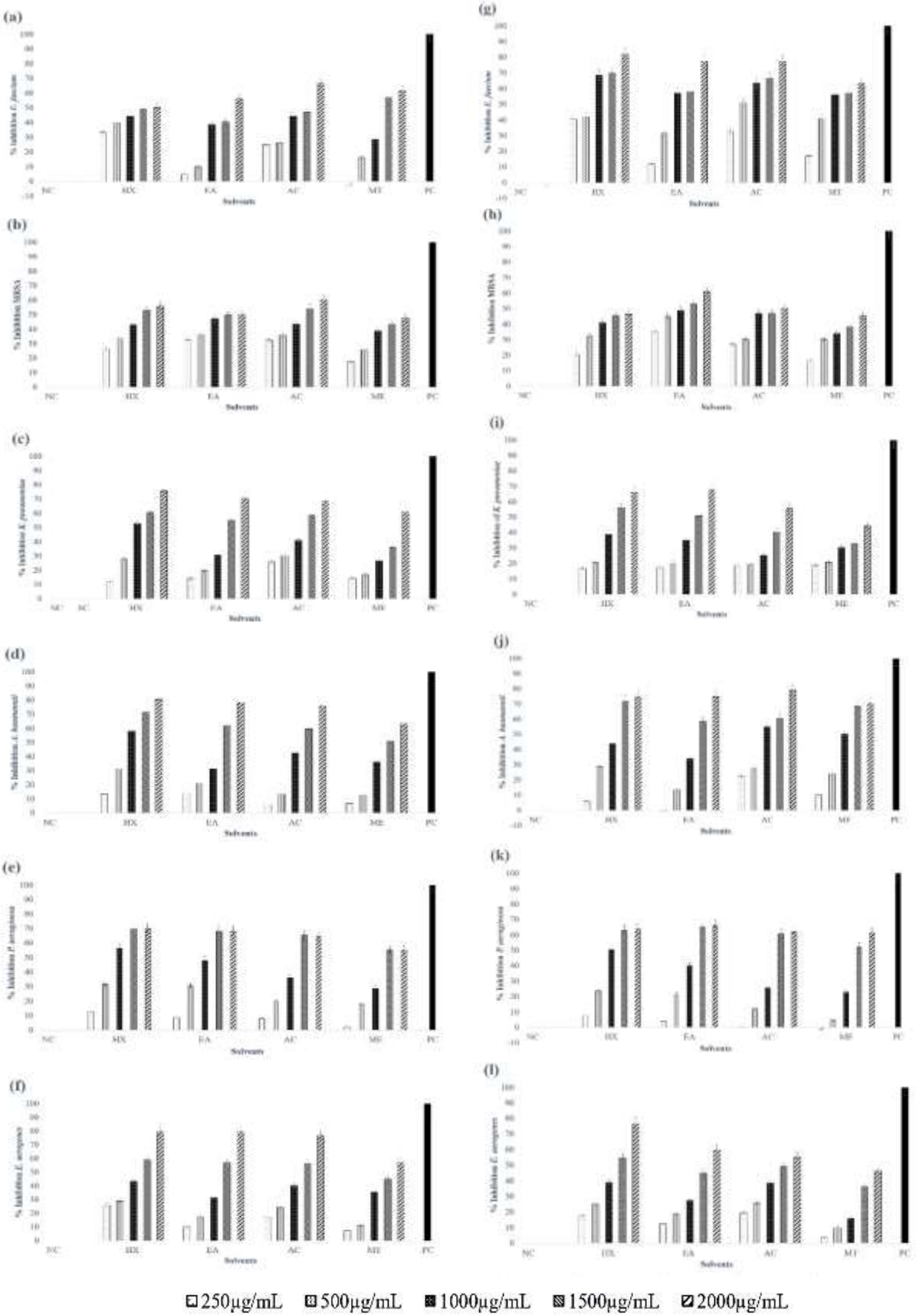

Figure 1| The minimum inhibitory concentration (MIC) of CEF of *Cuminum cyminum* (a, b, c, d, e, f) and *Foeniculum vulgare* (g, h, i, j, k, l) measured by the broth dilution method against ESKAPE pathogens. .
*NC = Negative control, HX = Hexane, EA = Ethyl acetate, AC = Acetone, ME = Methanol, PC = Positive control

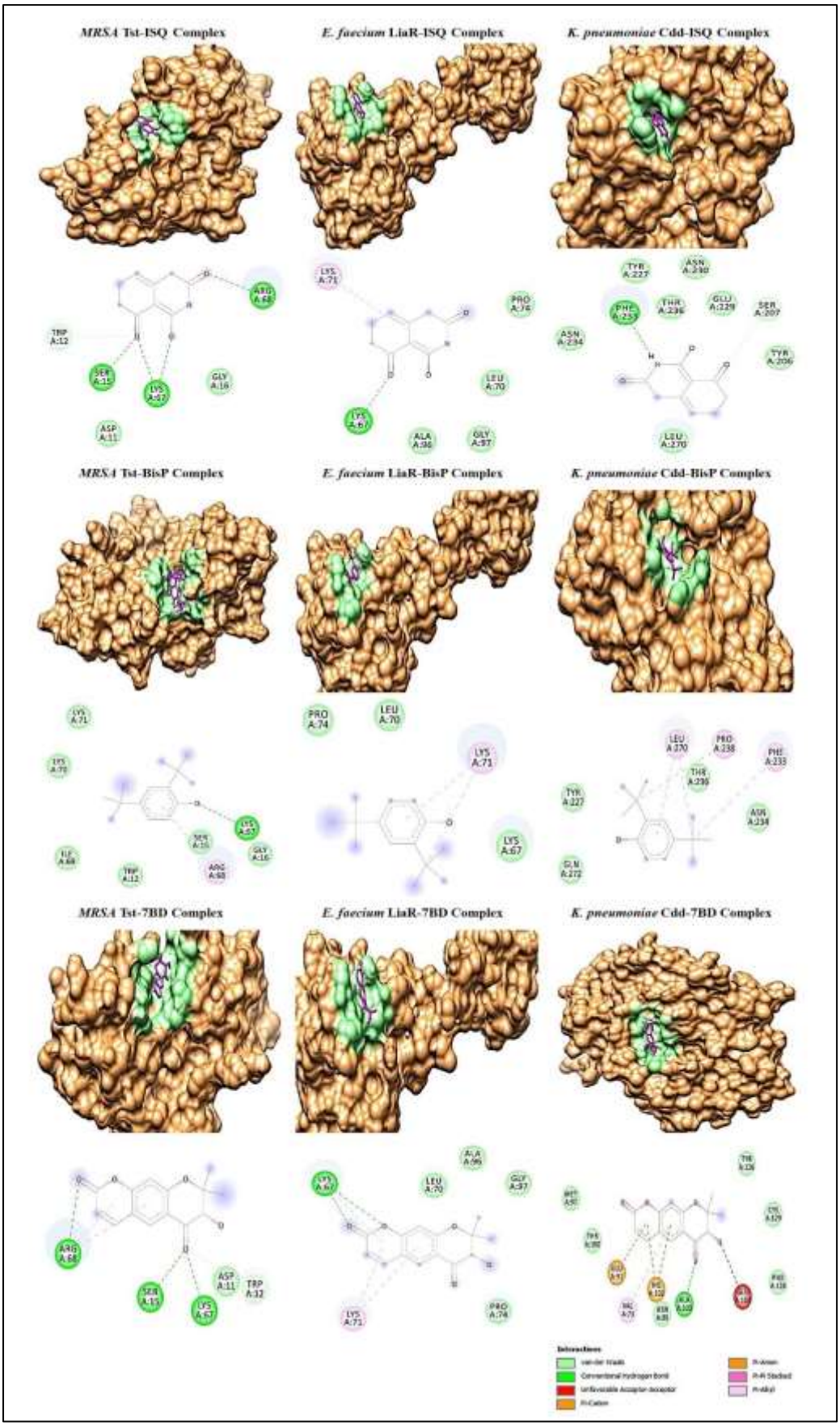

Figure 2. Three-dimensional (3D) illustration of 1,8(2H,5H)-Isoquinolinedione, 6,7-dihydro-3-hydroxy- (ISQ), Phenol, 2,4bis(1,1-dimethylethyl)- (BisP), and 2H,6H-Benzo[1,2-b:5,4-b']dipyran-2,6-dione, 7,8-dihydro-8,8-dimethyl- (7BD) binding with Tst, LiaR, and Cdd proteins of MRSA, *E. faecium*, and *K. pneumoniae* respectively. In addition, the localization of active residues with their type of interaction bonds have also been shown.

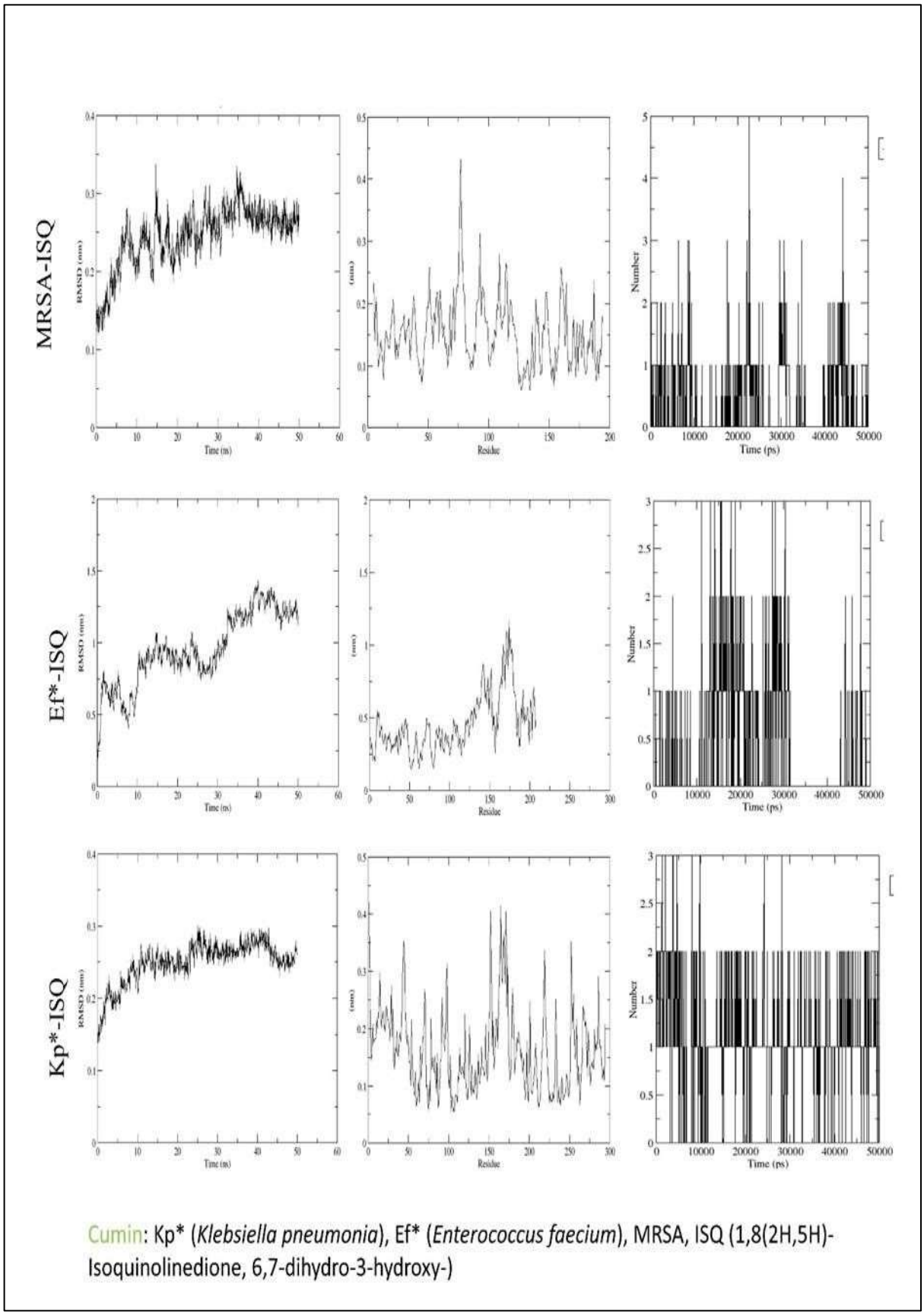

Figure 3| Receptor-ligand interactions over the period of MD simulation, of which (A) RMSD values, (B) RMS fuctuation-perresidue of receptor macromolecule, and (C) number of hydrogen bonds between receptor and ligand were computed over the course of MD. Note ISQ—(1,8(2H,5H)-Isoquinolinedione, 6,7-dihydro-3-hydroxy-), Ef*—*E. faecium,* and Kp*—*K. pneumoniae* respectively.

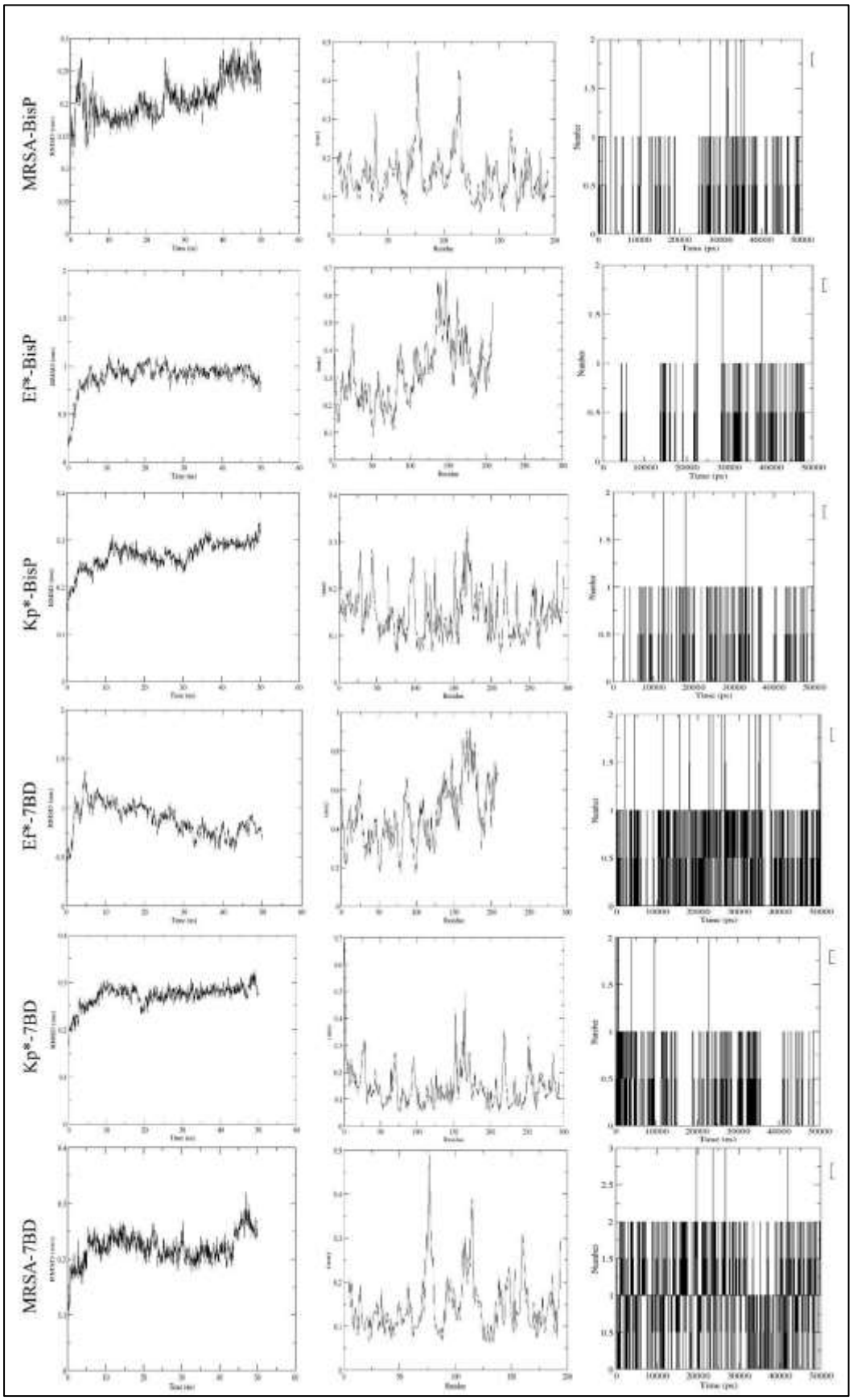

Figure 4| Receptor-ligand interactions over the period of MD simulation, of which (A) RMSD values, (B) RMS fuctuation-perresidue of receptor macromolecule, and (C) number of hydrogen bonds between receptor and ligand were computed over the course of MD. Note BisP—(Phenol, 2,4-bis(1,1-dimethylethyl)-), 7BD—(2H,6H- Benzo[1,2-b:5,4-b']dipyran-2,6-dione, 7,8-dihydro-8,8dimethyl-), Ef*—*E. faecium,* and Kp*—*K. pneumoniae* respectively.

VII. CONCLUSION:

The present study explores a strategic approach of unearthing natural product based bioactive as alternative drug repurposing agents for the treatment of MDR ESKAPE pathogens. A similar Apiaceae class-based set of two spices were utilised for the purpose of extracting their crude fractions in a series of four solvents. These crude extract fractions were tested against the MDR ESKAPE pathogens for their preliminary antibacterial activities. A stepwise approach of chromatographic separation was taken to unravel a multitude of chemical compounds for further virtual and pharmacological screening against selected virulent proteins of the ESKAPE pathogens. This helped in portraying classes of compounds like isoquinolinedione and coumarin derivatives with the potential for repurposing as antibacterial agents from natural sources. While a series of studies might be needed for exploring the drug repurposing capacities of these newly explored compounds, this study lays the foundation for upcoming researchers to utilize such approach for unearthing new potentials of already known natural products like cumin and fennel.

VIII. ACKNOWLEDGMENTS:

CL conceived the concepts, planned and designed the analyses. TM extracted the natural products and assessed their antibacterial activities which were furthered up by SP and PD. TM analyzed the data for GC-MS with the help of CL. MS conducted the computational studies with occasional inputs from CL. Figures and tables were generated by TM and MS with guidance provided by CL. TM and MS primarily wrote the draft manuscript aided by complete editorial upgradation by CL and formatting by SP and PD.

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