PN
PHYTONUTRIENTS

Information available at: infor@phytonutrients.pk
PHYTONutrients
Journal home page: https://phytonutrients.pk/index.php/pn

Lyceum Publisher

# The Green Solution: *In-Vitro* and *In-Silico* Screening of *Fagonia cretica* and *Berberis lyceum* Phytochemicals against *Haemophilus influenzae*

**Gul-e-Nayab[1]; Sadaf Niaz[1]; Shabir Ahmad[2]; Abid Ali[1]; Saghir Ahmad[3]; Ijaz Muhammad*[1 3*]**

[1]Department of Zoology Abdul Wali Khan University Mardan, Mardan, Pakistan.
[2]Department of Biotechnology Sarhad University of Science & Technology Peshawar Pakistan.
[3]Department of Microbiology Abdul Wali Khan University Mardan, Mardan, Pakistan.
*Correspondence: Ijaz Muhammad: E-mail: ijazmawkum@gmail.com

Received: April 2, 2024
Accepted: April 5, 2024
Published: July 21, 2024
Volume: 03: 2024
Article ID: pn.v3i.23
https://doi.org/10.62368/pn.v3i.23



**ABSTRACT:** *Haemophilus influenzae* (family Pasteurellaceae) is a coccobacillus, which is a non-spore-forming, non-motile, and non-acid-fast Gram-negative bacteria, present in upper respiratory tract causing epiglottitis, chronic bronchitis, meningitis, sinusitis, and community-acquired pneumonia. *H. influenzae* is resistant to certain antibiotics; novel drug discoveries are needed. In the present study, *B. lyceum* and *F. cretica* plant phytochemicals were tested *in vitro* against *H. influenzae.* Zones of inhibition were measured after the crude extract was tested using a well diffusion method against *H. influenzae* cultured on chocolate agar. Methanolic crude extract from *B. lyceum* showed a maximum zone of inhibition (32 mm) followed by ethanolic extract (30 mm). Similarly, methanolic extract from *F. cretica* showed maximum zone of inhibition (29 mm) followed by ethanolic extract (27.5 mm). Cytotoxic factor outer membrane protein (OMP) from *H. influenzae* was docked with the previously identified phytochemicals of these plants. *In silico* analysis shows that phytic acid from these plants is the most potent inhibitor of outer membrane protein (P6) followed by sindamine and karakuramine. The present study recommends that *B. lyceum* phytochemicals can be *synergistically* used as a potential drug against *H. influenzae.*

**Keywords:** *Fagonia cretica*; *Berberis lyceum*; *Haemophilus influenzae*; Green; OMP; Molecular Docking

## 1. Introduction

*H. influenzae* is a coccobacillus, which is a non-spore-forming, non-motile, and non-acid-fast Gram-negative bacteria belonging to the family Pasteurellaceae (Fleury et al., 2014; Hartwig, Ketterer, Apicella, & Varga, 2016; Ulanova & Tsang, 2014). Mostly observed in the upper respiratory tract, such as the oropharynx and nasopharynx of human beings, *H. influenzae* may colonize the genital and urinary mucosa,

resulting in neonatal urinary tract infection (UTI) and obstetric infections (Agrawal & Murphy, 2011; Geelen et al., 2014). Also, *H. influenzae* is responsible for epiglottitis, chronic bronchitis, meningitis, sinusitis, and community-acquired pneumonia and can act as an obligatory human parasite (Shahbaaz, Ahmad, & Hassan, 2015). Moreover, the major diseases caused by *H. influenza* are childhood pneumonia, meningitis bacteremia, community-acquired pneumonia (CAP) in adults, acute otitis media (AOM), acute sinusitis, and acute exacerbations of chronic bronchitis (AECB) (Hirano, Kodama, Kawano, & Suzuki, 2016).

Based on the presence or absence of a polysaccharide capsule, *H. influenzae* is categorized into two strains, the non-encapsulated and encapsulated strains commonly referred to as typeable and non-typeable *H. influenzae* (NTHi), respectively (Kostyanev & Sechanova, 2012). The encapsulated strain of *H. influenzae* is further classified into six diverse serotypes: a, b, c, d, e, and f. In contrast, the remaining non-encapsulated strain of *H. influenzae* is labeled as nontypeable *H. influenzae* (NTHi) (Barkatullah & Ibrar, 2011).

*H. influenzae* type b (Hib) is categorized on the base of capsular polysaccharide structure into two diverse genotypes, type I and type II (Khodashahri et al., 2015). *H. influenzae* type b (Hib), in combination with *Neisseria meningitides and Streptococcus pneumoniae*, can cause considerable aggressive infection and death in infants (Voysey, Sadarangani, Clutterbuck, Bolgiano, & Pollard, 2016). The encapsulated *H. influenzae* type b (Hib) is more cytotoxic and can lead to epiglottitis, meningitis, pneumonia, osteomyelitis, septic arthritis, and septicemia (Ulanova & Tsang, 2014; Zhu et al., 2015). The capsular polysaccharide serotype b is a major virulence factor, rendering *H. influenzae* resistant to phagocytosis and

helping the evasion of intracellular killing in neutrophils (Kaur, George, Pena-Ricardo, Kelly, & Watson, 2013; Kostyanev & Sechanova, 2012).

One of the major problems *controlling H. influenzae* infection is its sensitivity to resist some antibiotics, particularly ampicillin and other beta-lactams, tetracycline, trimethoprim, and sulfamethoxazole. The P6 is an outer membrane protein expressed on the surface of the encapsulated and nontypable strains. The gene that encodes P6 is highly preserved, which determines the high degree of similarity in terms of its nucleotide sequence in different strains (Kumar, Kumar, Navneet, & Gautam, 2014). Most of the studied *H. influenzae* strains *contain highly conserved P6 protein; therefore,* it has been suggested that this protein may be used as a potential vaccine (Michel et al., 2013). Several research groups have identified conserved surface proteins such as OMP (P6) and protein D and tested them as putative vaccines (Hua et al., 2016). However, controlling the infection transmitted by *H. influenzae is still a demand.*

Since immemorial, humans have used medicinal plants to treat various ailments (H. Ali, Uddin, & Jalal, 2015). Plants have antimicrobial agents used extensively as herbal drugs against different microbes (Iqbal, Ullah, Ibrahim, Ahmed, & Jabbar, 2015). *B. lyceum* (Berberidaceae) is a quickly growing herb used in traditional medicines to control various diseases (Gupta, Singh, & Joshi, 2015). On the other hand, *F. cretica* (*Zygophyllaceae)* is a tropical herb found in dry calcareous rocks throughout Pakistan, which contains significant phytochemicals well-known for medicinal uses (Iqbal et al., 2015).

This study aims to check the activity of *F. cretica* and *B. lyceum* extract against *H. influenzae* and compare the effect of these extracts with each other and with standard drugs, oxy-tetracycline dehydrate and doxycycline. Inhibitory activities of the phytochemicals of these plants were computationally proved through docking phytochemicals with virulence factor OMP (P6) of *H. influenzae* by molecular docking analysis.

## 2. Methodology

### 2.1. Ethical Approval

Ethical approval for this study was given by a member of the board of studies at the Department of Zoology, Abdul Wali Khan University Mardan.

### 2.2. Study Area and Sample Collection

Clinical isolates from patients were randomly collected from different hospitals of Khyber Pakhtunkhwa

(KP), including Mardan Medical Complex, District Head Quarter Hospital Mardan, District Head Quarter Hospital Swabi, Bacha Khan Medical Complex Swabi, Agha Khan Collection point during September 2015 to November 2016. Isolates were collected in aerobic culture bottles containing Tryptic Soy Broth (TSB) to enhance *H. influenzae* growth(Kim & Rhee, 2016). These TSB bottles were brought to the Parasitology Laboratory, Department of Zoology, Abdul Wali Khan University Mardan, to culture *H. influenzae*. TSB bottles containing clinical isolates were incubated at 37 °C for a minimum of 3 days and a maximum of up to 7 days. Chocolate agar was used as culture media for the growth of *H. influenzae* (Mukundan, Ecevit, Patel, Marrs, & Gilsdorf, 2008). A sterile environment was maintained to avoid contamination throughout the experiments.

**2.3. Colony confirmation**

*H. influenzae* colonies were obtained after incubation and further identified through a microscopic examination using a standard Gram staining procedure (Claus, 1992). After Gram staining, further confirmation of *H. influenzae* was made through urease, catalase, and oxidase tests according to the standard procedure described earlier (S. Ali et al., 2014; Paliwal & Randhawa, 1977).

**2.4. Plant Extract Preparation**

Selected plants *F. cretica* and *B. lyceum* were collected from different areas of KP in April 2016. Plants were collected carefully using different sharp cutters and identified in the Botany Department, Abdul Wali Khan University Mardan (AWKUM). Plant extracts were prepared with slight modifications according to the previous method, soil or any remaining particles were removed by shaking and thoroughly washed with distilled water (Edeoga, Okwu, & Mbaebie, 2005). The collected plants were dried in the shade for ten days at room temperature and ground into fine powder by tissue homogenizer. The powdered material was transferred into absolute methanol and ethanol (Merk, Germany) and extracted in the soxhlet extractor for 72 h. The extracts were filtered through a Whatman filter paper (125 mm) and concentrated using a rotary evaporator (Stuart, UK) with the water bath set at 40 °C. The powdered residue was transferred into vials and stored at 4 °C in airtight vials before analysis. Finally, 1 mg of *F. cretica* and *B. lyceum* crude extract was dissolved in 100 ml Dimethyl sulfoxide (DMSO).

**2.5. Application of Plant Extracts on *H. influenzae* Culture**

Antibacterial activity was assessed by the agar well diffusion method (Balouiri, Sadiki, & Ibnsouda, 2016), a colony of *H. influenzae* was taken and spread over chocolate agar with the help of cotton buds. The plant extracts were applied at different concentrations to freshly cultured *H. influenzae*. To apply plant extracts to culture media, 9 mm wells were bored in each plate with the help of a borer in culture media. Plant extracts at different concentrations were applied through a micro pipette in these wells. Two antibiotics, oxytetracycline dehydrate (Munawar Pharma, Pakistan) and doxycyclin (Mediceena Pharma, Pakistan) dissolved in DMSO (Merk, Germany), were used as control. The plates were then incubated at 37 °C for 24 hours. The diameter of the clear zones, showing no bacterial growth, around each well was measured. The activity was confirmed by measuring the diameter of the inhibition zone in millimeters (mm) (Muhammad, Niaz, et al., 2021). The mean clear zone of plates was calculated with standard deviation.

**2.6. Chemical Test for Detection of Compounds**

Biochemical tests were performed to determine the presence of various compounds in a crude extract of *F. cretica* and *B. lyceum*, according to the previously described (Tadesse, Ganesan, Nair, Letha, & Gani, 2016). For example, for the detection of alkaloids, 3-5 drops of Wagner's reagent (1.27 g of iodine and 2 g of potassium iodide in 100 ml of water) were added to the 3 ml portion of extract. Carbohydrates were observed by adding 2-3 drops of Molisch's reagent to 2 ml of extract and 2 ml of concentrated sulfuric acid. They left the mixture for two to three minutes. For the presence of glycosides, 2 ml of glacial acetic acid was added to 5 ml of extract, and one drop of ferric chloride solution was supplemented and gently underlaid with 1 ml of concentrated $H_2SO_4$. To confirm flavonoids, 2-3 drops of 20 % NaOH solution were added to

2 ml of extract. The presence of phenols was witnessed by adding 5 % aqueous ferric chloride to the 2ml crude extract. To confirm phlobatannins, 2 ml of extract was boiled with 1 ml of 1 % aqueous hydrochloric acid. For protein detection, 3-4 drops of ninhydrin solution were added to 2 ml of extract and kept in a boiling water bath for 1-2 minutes. For the existence of saponins, 6 ml of water was added to 2 ml of extract in a test tube and shaken well. Tannins were observed by adding 10 % alcoholic ferric chloride solution to 2 ml of crude extract. Terpenoids were followed by treatment of 2 ml extract with 1 ml of chloroform and a few drops of concentrated $H_2SO_4$. Quinones were observed by addition of 2-3 drops of concentrated hydrochloric acid to 3 ml extract. 3-4 drops of ethanoic acid glacial were added to 4 ml extract to check oxalates.

**2.7. Identification of *H. influenzae* Pathogenicity Factors**

Factors responsible for the pathogenicity of *H. influenzae* were identified by scanning the literature and used as drug targets in the current study. The sequences of OMP (P6) were retrieved from the Database of the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov). The retrieved sequence in FASTA format was used in BLAST query using BLASTP (version 2.7.0) to retrieve homologous protein sequences from closely related species including *E. coli* str. k-12 substr. mg1655, *S. oneidensis* MR-1, *P. aeruginosa* PAO1, *N. meningitidis* MC58. Proteins were aligned using the multiple alignment sequencing tool Clustal OMEGA v. 1.2.4. (http://www.ebi.ac.uk/Tools/msa/ clustalo/). The structure of OMP (P6) PDB ID **(2AIZ)** was retrieved from the Research Collaboratory for Structural Bioinformatics protein data bank (RCSB PDB) in PDB format.

**2.8. Ligand preparation**

A deep literature survey identified 31 compounds from *B. lyceum* and *F. cretica* plants. Molecular structures of the ligands were drawn on chem-Draw ultra version 10.0. (2010), 3D protonation and energy were minimized for docking with the target protein while using MOE.

**2.9. Molecular Docking and Dynamic Simulation**

Structures of different phytochemicals were drawn using ChemDraw and saved in mol form in mdb format. Phytochemicals identified from selected plants and pathogenic factor OMP (P6) from *H. influenzae* were docked by using a Molecular Operating Environment (MOE), (v. 2014.0901) (Muhammad et al., 2022; Muhammad, Rahman, et al., 2021). Protonate 3D and energy minimization were performed through MOE. Protein-ligand docking score, ligand properties, and 2D and 3D structures were saved.

MOE performed molecular dynamic simulation. Before starting the simulation, proteins were solvated using water as a solvent. For simulation, the system was heated from 0 to 300 $^{o}$K, followed by equilibration for 100 ps and production for 600 ps. NPA (Nose-Poincare-Andersen) algorithm and Amber99 force field were used for 0.5 seconds per sample.

**3. Results**

**3.1. Confirmation of *H. influenzae* Culture**

Clinical isolates of *H. influenzae* collected from hospitals and cultured on chocolate agar for 24 hours appeared as shown in Fig 1. Firstly, *H. influenzae* was identified by colony morphology followed by Gram staining procedure, which shows the rod shape of bacteria. Further confirmation was performed through biochemical tests, which showed bubbles formation on the slide by adding $H_2O_2$ to bacterial colonies, indicating the presence of *H. influenzae*. On the reaction of *H. influenzae* with oxidase reagent, the color of the oxidase reagent changes to blue, and on culturing on urease media, the color of urease slants changed from orange to pink. **3.2. Application of Plant Extract to *H. influenzae* Culture**

Plant Crude extract showed activity by forming different inhibition zones at different concentrations (Fig. 2). The activity of each concentration was checked in triplicate form. For this purpose, four wells were made in each culture plate, and the same concentration was applied to two wells by dividing the plate into four equal parts. The activity of *F. cretica* and *B. lyceum* crude extracts were checked against *H. influenzae* at 2, 2.5, 4, and 5 µg/ml concentrations, respectively. In both extracts, the lowest inhibitory activity was observed

a

**Fig. 1. *H. influenzae* colonies on chocolate agar.**

at 2µg/ml concentration, and the highest activity was shown at 5 µg/ml concentration. *F. cretica* methanolic extract showed the lowest inhibition of (23 mm) at 2 µg/ml and the highest inhibition of (29 mm) at 5 µg/ml. Ethanolic extract of *F. cretica* showed the lowest inhibition (20.5 mm) at 2 µg/ml and the highest inhibition (27.5 mm) at 5 µg/ml. *B. lyceum* methanolic crude extract showed the lowest inhibition of (25 mm) at 2 µg/ml and the highest inhibition of (32 mm) at 5 µg/ml. Ethanolic extract of *B. lyceum* showed the lowest inhibition of (22.5 mm) at 2 µg/ml and the highest activity of (30 mm) at 5 µg/ml. By comparing all extracts, the lowest activity was shown by *F. cretica* (ethanol) of (20.5 mm) at 2 µg/ml, and the highest activity was shown by methanolic extract of *B. lyceum* at 5 µg/ml concentration was (32 mm) (Table 1).

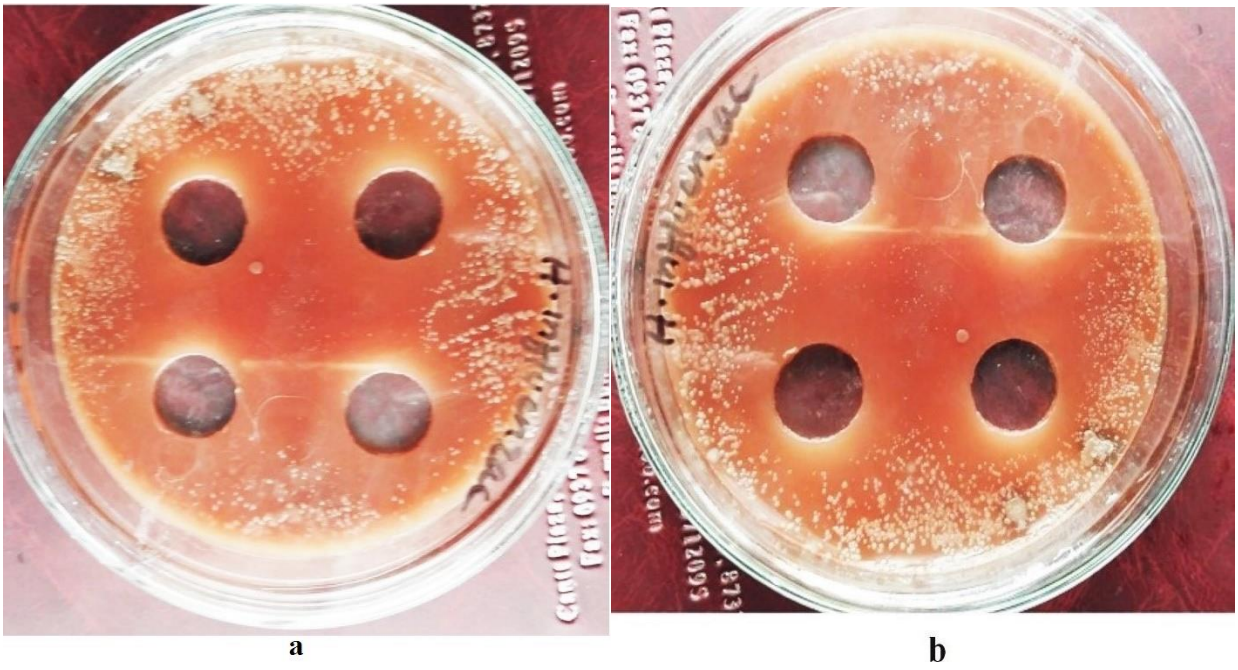
**3.3. Biochemical Test for Phytochemicals Detection**

*F. cretica* and *B. lyceum* were tested for primary metabolites responsible for the plant's biological activity. Tests performed for the presence of compounds such as alkaloids, carbohydrates, glycosides, flavonoids, phenols, phlobatannins, proteins, saponins, oxalate, tannins, terpenoids, and quinones are shown (Table 2). Both plants were negative for proteins and oxalate. Ethanol *F. cretica* was found negative for flavonoids. Phlobatannins were detected only in the ethanolic extract of *F. cretica.* Terpenoids were absent in the ethanolic extract of *F. cretica.*

**Table 1. Zone of inhibition shown in 'mm' of different plants extract and control at different concentrations against *H. influenzae*.**

| Extract | Concentration | | | | Mean±SEM |
|---|---|---|---|---|---|
| | 2 µg/ml | 2.5 µg/ml | 4 µg/ml | 5 µg/ml | |
| Methanol *F. cretica* | 23 | 25.75 | 27.5 | 29 | 26.31±1.288 |
| Ethanol *F. cretica* | 20.5 | 21 | 24.25 | 27.5 | 21.92±1.176 |
| Methanol *B. lyceum* | 25 | 26 | 29 | 32 | 28.00±1.581 |

| | | | | | |
|---|---|---|---|---|---|
| Ethanol *B. lyceum* | 22.5 | 25 | 27 | 30 | 26.13±1.586 |
| **Positive control** | | | | | |
| Doxycycline | 28 | 31 | 35 | 40 | 33.50±2.598 |
| Oxy-tetracycline dehydrate | 25 | 28 | 32 | 36 | 30.25±2.394 |
| **Negative Control** | | | | | |
| DMSO | No antibacterial activity | | | | |



**a** **b**

**Fig 2. (a) Zone of inhibition by *F. cretica* and (b) inhibition zone by *B. lyceum* extracts.**

**Table 2.** Presence of compounds in extracts of *B. lyceum* and *F. cretica.*

| Test | **Ethanol *F. cretica*** | **Methanol *F. cretica*** | **Ethanol *B. lyceum*** | **Methanol *B. lyceum*** |
|---|---|---|---|---|
| Alkaloids | + | + | + | + |
| Carbohydrates | + | + | + | + |
| Glycosides | + | + | + | + |
| Flavonoids | - | + | + | + |
| Phenol | + | + | + | + |
| Phlobatannins | + | - | - | - |
| Proteins | - | - | - | - |
| Saponins | + | + | + | + |
| Tannins | + | + | + | + |

| | | | | |
|---|---|---|---|---|
| Terpenoids | - | + | + | + |
| Quinones | + | + | + | + |
| Oxalate | - | - | - | - |

Alignment result shows (53, 40, 41 and 37 %) sequence similarities of Outer membrane protein P6 of *H. influenzae* with closely related species of *E. coli* str. k-12 substr. mg1655, *S. oneidensis* MR-1, *P. aeruginosa* PAO1, and *N. meningitidis* MC58 respectively.

**3.5. Molecular Docking**

Ligands were docked with *H. influenzae* target protein (OMP P6) using MOE to find the inhibitor of OMP P6. Docked results revealed that Phytic acid is the most potent inhibitor because it binds to OMP6 at different amino acids, such as Asn79, Arg73, Tyr78, and As971, with the highest energy of -16.3503. Sindamine was the second most potent inhibitor that potentially binds with amino acids such as Asn79, His119, Arg73, and Thr635, with a binding score of -13.7126 (Table 3). Structures of the potent ligands are shown in Fig 3.

**Table 3.** Docking score, rmsd of the screened ligand with OMP (P6) protein from *H. influenzae.*

| Compounds | Docking Score | rmsd-refine |
|---|---|---|
| 4-methyl-7-hydroxy coumarin | -9.9601 | 1.4647 |
| Gilgitine | -12.4491 | 3.5787 |
| Butyl-3-hydroxypropyl phthalate | -10.3051 | 2.9974 |
| Jatrorrhizine | -10.8918 | 2.6887 |
| Jhelumine | -10.3666 | 3.4087 |
| Methyl Triacontanoate | -9.5343 | 4.5968 |
| Octasonic Acid | -11.4625 | 4.4410 |
| Oleanolic Aldehyde Acetate | -6.2585 | 3.5315 |
| Arjunolic Acid | -11.9126 | 3.4302 |
| Oxyberberin | -11.1144 | 2.1668 |
| Oxyacanthine | -10.5814 | 1.7591 |
| Phytic Acid | -16.3503 | 2.0877 |
| Sindamine | -13.7126 | 2.8009 |
| Karakoramine | -13.0908 | 2.3644 |
| Vitamin A | -9.3068 | 2.0742 |
| β-Amyrene Acetate | -9.3003 | 4.7822 |
| 3-α, 23- Dihydroxyurs -12-en-28-Oic Acid | -10.1820 | 1.6235 |
| Kaemferole | -11.6211 | 0.9783 |
| Balochistanamine | -11.2011 | 2.5613 |
| Linoleic Acid | -7.5822 | 2.2321 |
| Palmatine | -10.8448 | 1.7473 |
| Punjabine | -12.5745 | 1.9772 |
| Quercitine | -10.2517 | 3.1381 |
| Stigmasterole | -10.5978 | 1.7392 |
| Triacontanoic Acid | -9.8971 | 6.9183 |
| β-sitosterole | -9.3034 | 3.9584 |
| 4, 4-dimethylhexadeca -3-ol | -9.9220 | 2.4189 |
| 3-(4'-(6-methyl- butyl) phenyl) propane- 1-ol | - 8.6945 | 2.2006 |
| Berbamunine | -11.2166 | 2.8420 |
| Berbamine | -8.4020 | 6.6087 |
| Berberine | -10.8847 | 3.7826 |

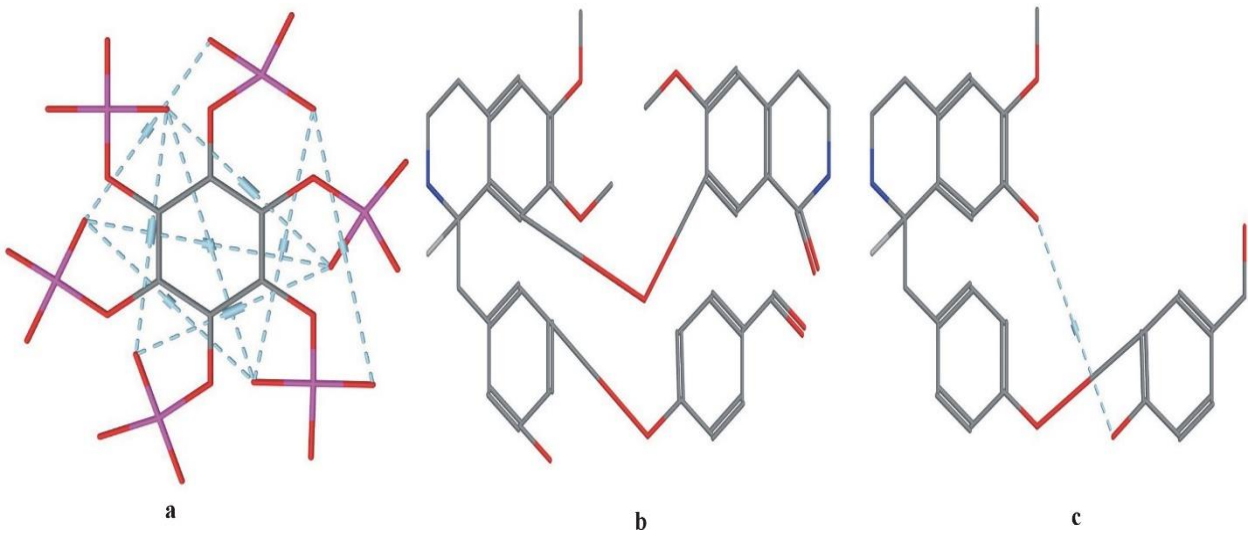



**Fig. 3. A.** 2D structure of the most potent and effective ligand against target protein (a). phytic acid (b) sindamine (c) karakuramine.

Phytic acid shows Acidic interaction with Asn79, Arg73, Tyr78, Asp71, and Thr75. Polar interaction was shown with Asp37, Asp71, Tyr78, Arg73, and Asn79. Greasy interaction was shown with amino acids Arg73, Asp37, Gly35, and Gly74. Basic interaction was shown with Gly74, Asp37, Phe36, and Asn79. Metal/ion contact was shown with the amino acid Arg73 (Fig. 4).

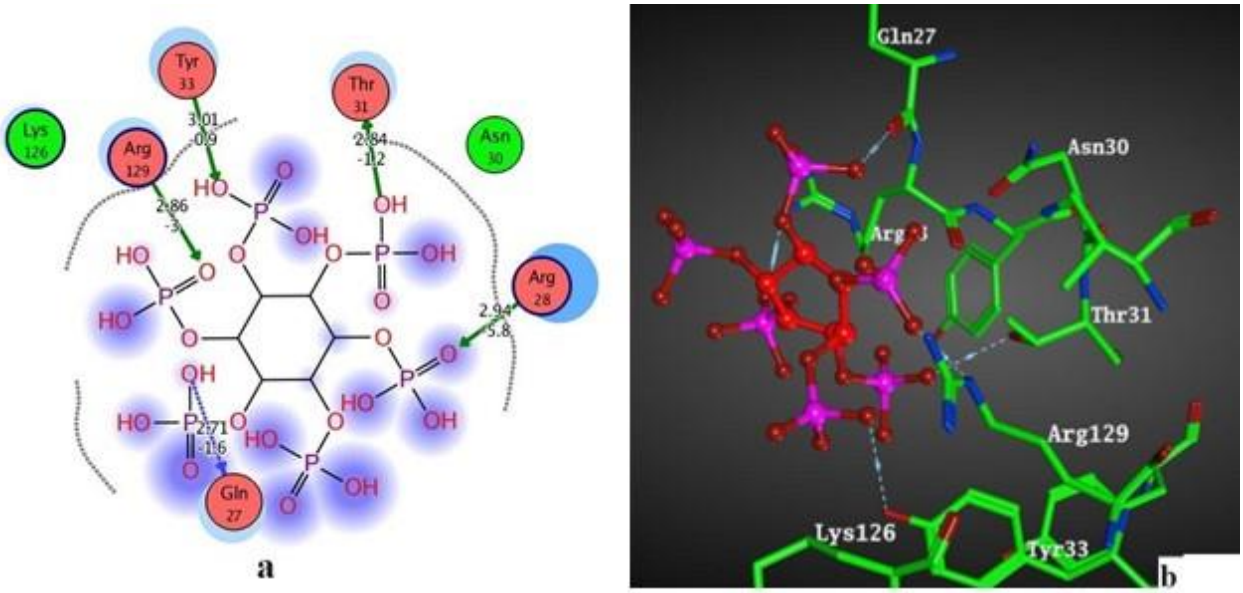



**Fig. 4. (a and b) show 2D and 3D interaction of phytic acid with outer membrane (P6) protein.**

Sindamine shows polar hydrogen interaction with amino acid Asn79. His119 shows Greasy hydrogen interaction. Acidic interaction was found with amino acid Arg73, and Arene-Arene interaction was found with Thr78 (Fig. 5).

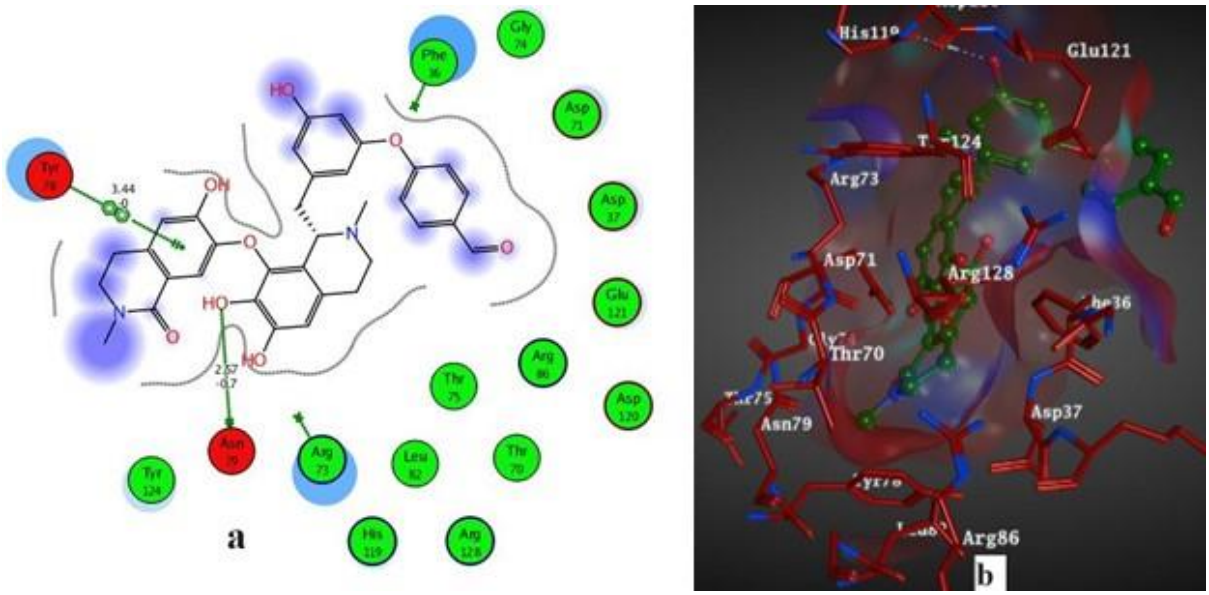



**Fig. 5.** a,b shows the 2D and 3D interaction of sindamine with outer membrane (P6) protein.

Karakuramine shows acidic interaction with Arg86. Polar interaction was shown with amino acids Asp37, Asp71, and Asn79, while basic interaction was shown with Asp71 and Asp37. The greasy interaction was shown with amino acids Gly35, Arg73, and Arene-Arene interaction with Tyr78 (Fig. 6).

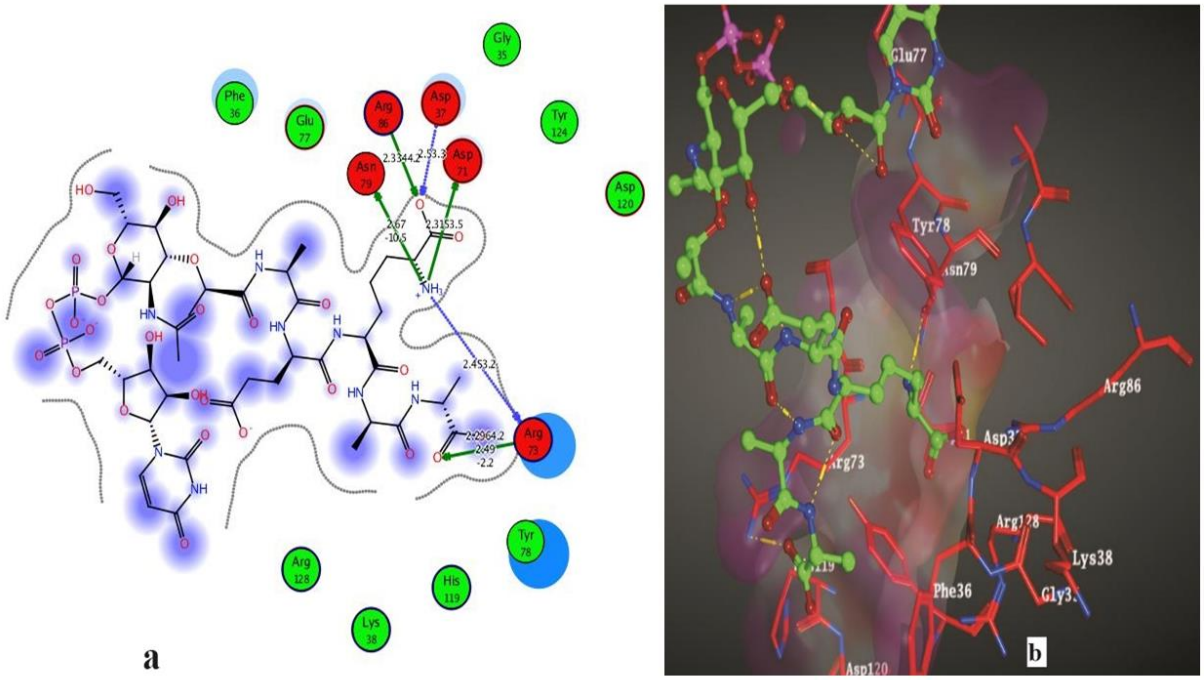



**Fig. 6.** (a,b) shows the 2D and 3D interaction of karakuramine with the outer membrane P6 protein.

### 3.6. Molecular Dynamic Simulation

Molecular dynamic simulation showed the kinetic and potential energy, instantaneous temperature, volume, and pressure of the molecule present in OMP (P6) of *H. influenzae* when the system was heated for a given period. The results of the molecular dynamic simulation are shown in Fig. 7.

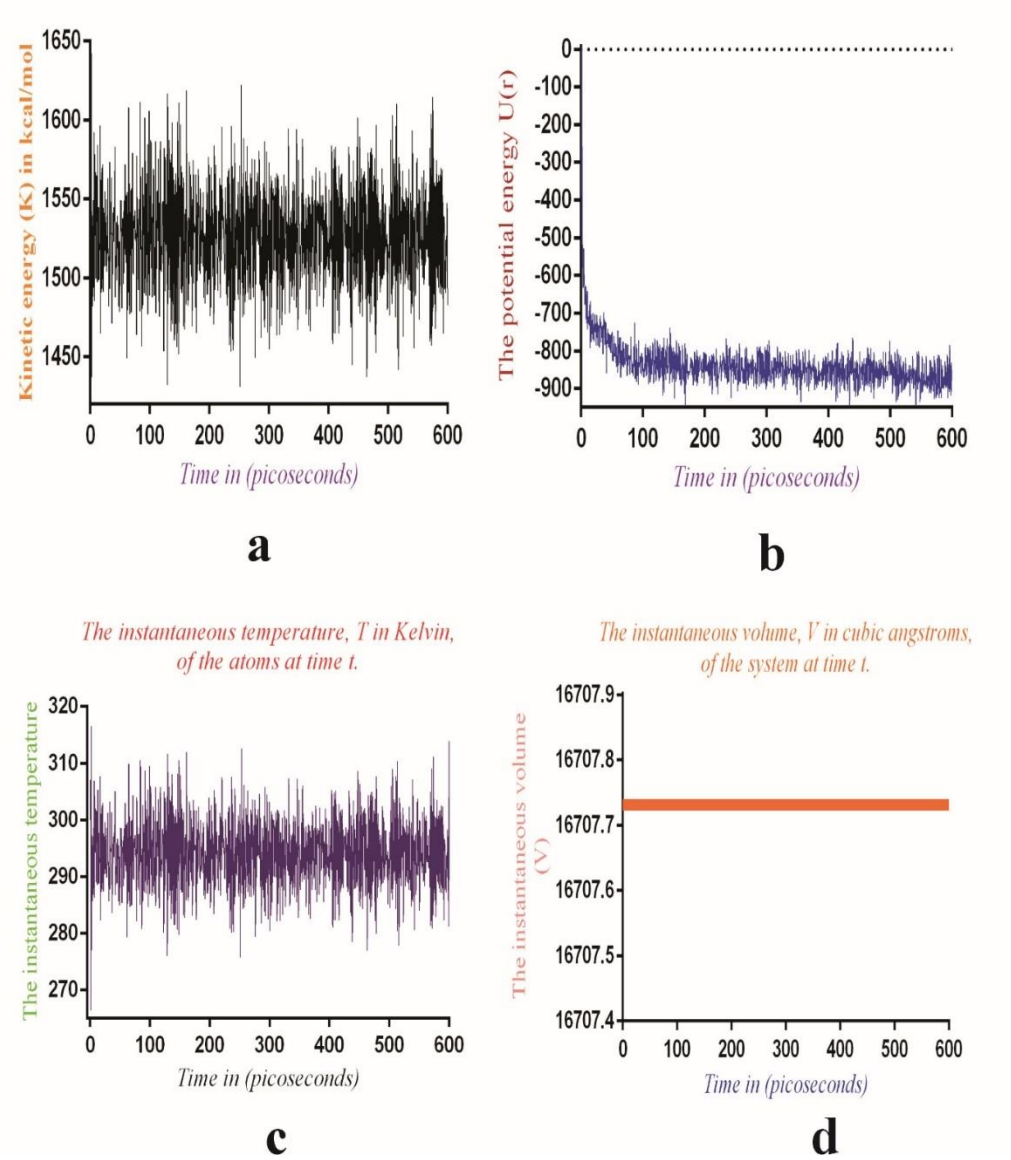



**Fig. 7.** (a, b) shows kinetic and potential energies; (c) shows variation in the molecule's temperature when haeted the system for about 600ps at constant volume, as shown in d.

### 4. Discussion

The resistance of *H. influenzae* to commonly used antimicrobials has risen in recent years, and wide variations exist in antimicrobial susceptibility rates spatiotemporally (Zhu et al., 2015). The present study was designed to check the inhibitory activity of *F. cretica* and *B. lyceum* extract against *H. influenzae.* Methanolic extracts of *F. cretica* showed the highest inhibitory activity (29 mm) at 5 µg/ml concentration and the lowest (23 mm) at 2 µg/ml. Ethanolic extract of *F. cretica* shows (27.7 mm) inhibition at 5 µg/ml and (20.5 mm) inhibition at 2 µg/ml. Differences in the inhibitory activity at the same concentration may be due to the differences in the phytochemical profiles of the plants. Doxycycline and oxy-tetracycline dehydrate antibiotics were used as positive controls, and both antibiotics showed the highest inhibitory activity compared to plant extracts. When the results were compared with the inhibitory activity of *C. rotundus* Linn, root extracts (Kumar et al., 2014) against *H. influenzae, a*

methanolic extract showed inhibitory activity of (18 mm) less than our findings. The difference may be due to the different phytochemicals found in these plants.

A comparison of results obtained from *F. cretica* methanolic and ethanolic extract with methanolic and ethanolic extract of *B. lyceum* root showed that methanolic root extract of *B. lyceum* provides (32 mm) zone of inhibition at 5 µg/ml and (25 mm) zone of inhibition at 2 µg/ml concentration while ethanolic extract of *B. lyceum* root shows (30 mm) zone of inhibition at 5 µg/ml and (22.5 mm) zone of inhibition at 2 µg/ml concentration. No significant differences were observed between methanolic and ethanolic extracts at 5 µg/ml concentration; however, variations were observed at 2 µg/ml concentration. When the activity of both plants was compared, *B. lyceum* extract possessed the highest inhibitory activity compared to *F. cretica.* The differences in inhibitory activity may be due to the difference in the phytochemical profile of both plants. According to (Kumar et al., 2014), acetone extract of *L. vulgaris* Ser. shows (16 mm), and methanolic. *H. influenzae* is not known to produce protein toxins but can interact with their host by OMPs, capsules, fimbrial adhesin, Lipooligosaccharide, and Protein D. In the present study, an OMP (P6) was selected because it has been characterized as conserved among all *H. influenzae* strains (Agrawal & Murphy, 2011; Rosadini, 2011). To prove the obtained *in vitro* results by an *in silico* study, phytochemicals of both plants available online were identified and docked with the OMP (P6) protein of *H. influenzae.* Among docked compounds, phytic acid showed maximum binding energy (-16.350 kcal/mol), followed by sindamine and karakuramine; studies have shown that phytic acid is a natural phytochemical occurring in plants that exhibits antibacterial activity and is also safe for human use (Nassar & Nassar, 2016). Also, combined treatment of nisin–phytic acid and nisin–pediocin–phytic acid caused substantial declines of pathogenic bacteria (*L. monocytogenes*) in cabbage and broccoli and can be used as a potential antimicrobial agent (Bari et al., 2005) and anti-cancer activity (Muhammad et al., 2020). Moreover, a recent study showed that phytic acid and NaCl act synergistically to destroy *E. coli* cells protected by a biofilm (Blasdell et al., 2016).

Sindamine and karakuramine activity against *H. influenzae* has never been investigated. However, our *in-silico* results showed that these compounds may be used as novel potential drugs for treating *H. influenzae* infection. **5. Conclusions**

The study investigated *in vitro* and *in silico* screening of *F. cretica* and *B. lyceum* as a potential drug against *H. influenzae*. Methanolic and ethanolic extracts of *F. cretica* and *B. lyceum* were tested against *H. influenzae.* Methanolic extract of *B. lyceum* showed a maximum (32 mm) zone of inhibition, followed by ethanolic extract, which showed a maximum (30 mm) zone of inhibition at 5 µg/ml. Methanolic extract *F. cretica* shows (a 29 mm) zone of inhibition, followed by ethanolic extract, which shows (a 27.5 mm) zone of inhibition at 5 µg/ml. The minimum zone of inhibition (20.5 mm) was shown by ethanolic extract of *F. cretica* at 2 µg/ml followed by ethanolic extract of *B. lyceum,* which showed (22.5 mm) zone of inhibition at 2 µg/ml. Docking analysis proved the obtained *in vitro* result by showing that phytic acid present in *B. lyceum* is the most potent inhibitor, followed by sindamine and karakuramine, and these compounds can be used as potent anti-*H. influenzae* drugs.

**Data Availability:** The data and materials supporting the conclusions of this article are included within the article.

**Authors contribution**: Gul-e-Nayab, Shabir Ahmad, Saghir Ahmad, and carried out the experimental and written the initial draft of the manuscript. Ijaz Muhammad Sadaf Niaz and Abid Ali designed and supervised the overall study.

**Conflicts of Interest:** The authors have no conflicts of interest to declare.

**Funding statement:** More specific funding is needed for this work.

**References**

Agrawal, A., & Murphy, T. F. (2011). Haemophilus influenzae infections in the H. influenzae type b conjugate vaccine era. *Journal of clinical microbiology*, *49*(11), 3728-3732.

Ali, H., Uddin, S., & Jalal, S. (2015). Chemistry and biological activities of Berberis lycium Royle. *Journal of Biologically Active Products from Nature, 5*(5), 295-312.

Ali, S., Ali, Q., Melzer, F., Khan, I., Akhter, S., Neubauer, H., & Jamal, S. M. (2014). Isolation and identification of bovine Brucella isolates from Pakistan by biochemical tests and PCR. *Tropical animal health and production, 46*, 73-78.

Balouiri, M., Sadiki, M., & Ibnsouda, S. K. (2016). Methods for in vitro evaluating antimicrobial activity: A review. *Journal of pharmaceutical analysis, 6*(2), 71-79.

Bari, M., Ukuku, D., Kawasaki, T., Inatsu, Y., Isshiki, K., & Kawamoto, S. (2005). Combined efficacy of nisin and pediocin with sodium lactate, citric acid, phytic acid, and potassium sorbate and EDTA in reducing the Listeria monocytogenes population of inoculated

fresh-cut produce. *Journal of food protection, 68*(7), 1381-1387.

Barkatullah, B., & Ibrar, M. (2011). Plants profile of malakand pass hills, district malakand, Pakistan. *African Journal of Biotechnology, 10*(73), 16521-16535.

Blasdell, K. R., Duong, V., Eloit, M., Chretien, F., Ly, S., Hul, V., . . . Buchy, P. (2016). Evidence of human infection by a new mammarenavirus endemic to Southeastern Asia. *Elife, 5*, e13135.

Claus, D. (1992). A standardized Gram staining procedure. *World journal of Microbiology and Biotechnology, 8*, 451-452.

Edeoga, H. O., Okwu, D., & Mbaebie, B. (2005). Phytochemical constituents of some Nigerian medicinal plants. *African journal of biotechnology, 4*(7), 685-688.

Fleury, C., Su, Y.-C., Hallström, T., Sandblad, L., Zipfel, P. F., & Riesbeck, K. (2014). Identification of a Haemophilus influenzae factor H–binding lipoprotein involved in serum resistance. *The Journal of Immunology, 192*(12), 5913-5923.

Geelen, T. H., Gaajetaan, G. R., Wouters, E. F., Rohde, G. G., Franssen, F. M., Grauls, G. E., . . . Stassen, F. R. (2014). The host immune response contributes to Haemophilus influenzae virulence. *Respiratory Medicine, 108*(1), 144-152.

Gupta, M., Singh, A., & Joshi, H. C. (2015). Berberis Lycium multipotential medicinal application: An overview. *Int. J. Chem. Stud, 3*(4), 10-13.

Hartwig, S. M., Ketterer, M., Apicella, M. A., & Varga, S. M. (2016). Non-typeable Haemophilus influenzae protects human airway epithelial cells from a subsequent respiratory syncytial virus challenge. *Virology, 498*, 128-135.

Hirano, T., Kodama, S., Kawano, T., & Suzuki, M. (2016). Accumulation of regulatory T cells and chronic inflammation in the middle ear in a mouse model of chronic otitis media with effusion induced by combined eustachian tube blockage and nontypeable Haemophilus influenzae infection. *Infection and immunity, 84*(1), 356-364.

Hua, C.-Z., Hu, W.-L., Shang, S.-Q., Li, J.-P., Hong, L.-Q., & Yan, J. (2016). Serum concentrations of antibodies against outer membrane protein P6, protein D, and T-and B-cell combined antigenic epitopes of nontypeable Haemophilus influenzae in children and adults of different ages. *Clinical and Vaccine Immunology, 23*(2), 155-161.

Iqbal, M., Ullah, N., Ibrahim, M., Ahmed, U., & Jabbar, A. (2015). In-Vitro Antibacterial Study of Fagonia Cretica. *In-Vitro*(4), 6-9.

Kaur, I., George, K. J., Pena-Ricardo, C., Kelly, B. A., & Watson, B. (2013). Association of postpartum maternal tetanus toxoid, reduced diphtheria toxoid and acellular pertussis vaccine administration and timeliness of infant immunization. *The Pediatric infectious disease journal, 32*(11), 1287-1289.

Khodashahri, S. B., Siadat, S. D., Rahbar, M., Abdollahpour-Alitappeh, M., Vaziri, F., Rahnamaye-Farzami, M., . . . Masoumi, M. (2015). Genotyping of Haemophilus influenzae type b strains and their incidence in the clinical samples isolated from Iranian patients. *Iranian Journal of Microbiology, 7*(3), 136.

Kim, N. H., & Rhee, M. S. (2016). Phytic acid and sodium chloride show marked synergistic bactericidal effects against nonadapted and acid-adapted Escherichia

coli O157: H7 strains. *Applied and environmental microbiology, 82*(4), 1040-1049.

Kostyanev, T. S., & Sechanova, L. P. (2012). Virulence factors and mechanisms of antibiotic resistance of Haemophilus influenzae. *Folia Med (Plovdiv), 54*(1), 19-23.

Kumar, S., Kumar, K., Navneet, D., & Gautam, S. S. (2014). Antibacterial evaluation of Cyperus rotundus Linn. root extracts against respiratory tract pathogens. *African Journal of Pharmacology and Therapeutics, 3*(3).

Michel, L. V., Snyder, J., Schmidt, R., Milillo, J., Grimaldi, K., Kalmeta, B., . . . Pichichero, M. E. (2013). Dual orientation of the outer membrane lipoprotein P6 of nontypeable Haemophilus influenzae. *Journal of bacteriology, 195*(14), 3252-3259.

Muhammad, I., Nayab, G. E., Rahman, N., Niaz, S., Ali, A., & Khan, H. (2022). Anticancer Potential of Diospyros kaki (persimmon) polyphenols against AKT1 (6CCY): Docking based in silico study. *PHYTONutrients*, 57-68.

Muhammad, I., Niaz, S., Nayab, G. E., Hussain, A., Ahmad, S., Rahman, N., . . . Ali, A. (2021). Molecular Docking and In vitro Analysis of Fagonia Cretica and Berberis Lyceum Extracts Against Brucella Melitensis. *Current Computer-Aided Drug Design, 17*(7), 946-956.

Muhammad, I., Rahman, N., Nayab, G. E., Niaz, S., Shah, M., Afridi, S. G., . . . Capanoglu, E. (2020). The molecular docking of flavonoids isolated from Daucus carota as a dual inhibitor of MDM2 and MDMX. *Recent Patents on Anti-Cancer Drug Discovery, 15*(2), 154-164.

Muhammad, I., Rahman, N., Niaz, S., Basharat, Z., Rastrelli, L., Jayanthi, S., . . . Khan, H. (2021). Screening of potent phytochemical inhibitors against SARS-CoV-2 protease and its two Asian mutants. *Computers in Biology and Medicine, 133*, 104362.

Mukundan, D., Ecevit, Z., Patel, M., Marrs, C. F., & Gilsdorf, J. R. (2008). Pharyngeal colonization dynamics of Haemophilus influenzae and haemophilus haemolyticus in healthy adult carriers. *Journal of clinical microbiology, 46*(4), 1575.

Nassar, R. I., & Nassar, M. (2016). Antimicrobial effect of phytic acid on Enterococcus faecalis. *The International Arabic Journal of Antimicrobial Agents, 6*(4).

Paliwal, D., & Randhawa, H. (1977). Rapid method for detection of urea hydrolysis by yeasts. *Applied and environmental microbiology, 33*(2), 219-220.

Rosadini, C. V. (2011). *Roles of Secreted Virulence Factors in Pathogenicity of Haemophilus Influenzae: A Dissertation.*

Shahbaaz, M., Ahmad, F., & Hassan, M. I. (2015). Structure-based function analysis of putative conserved proteins with isomerase activity from Haemophilus influenzae. *3 Biotech, 5*, 741-763.

Tadesse, S., Ganesan, K., Nair, S. K. P., Letha, N., & Gani, S. B. (2016). Preliminary phytochemical screening of different solvent extracts of leaves and stems of Commelina benghalensis L.(Family: Commelinaceae). *Int. J. Pharm. Chem. Biol. Sci, 6*, 103-107.

Ulanova, M., & Tsang, R. S. (2014). Haemophilus influenzae serotype a as a cause of serious invasive infections. *The Lancet infectious diseases, 14*(1), 70-82.

Voysey, M., Sadarangani, M., Clutterbuck, E., Bolgiano, B., & Pollard, A. J. (2016). The impact of administration of conjugate vaccines containing cross reacting material on Haemophilus influenzae type b antibody responses in infants: A systematic review and meta-analysis of randomised controlled trials. *Vaccine, 34*(34), 3986-3992.

Zhu, H., Wang, A., Tong, J., Yuan, L., Gao, W., Shi, W., . . . Yang, Y. (2015). Nasopharyngeal carriage and antimicrobial susceptibility of Haemophilus influenzae among children younger than 5 years of age in Beijing, China. *BMC microbiology, 15*, 1-6.