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Lilium philadelphicum flower as a novel source of antimicrobial agents: A study of bioactivity, phytochemical analysis and partial identification of antimicrobial metabolites

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Abstract: The members of the Liliaceae family have been regarded as an excellent source of biologically active compounds. However, the work on antimicrobial potential and characterization of the bioactive fractions of *Lilium philadelphicum* flower is limited and needs to be explored. The present study reports the antimicrobial potential, anti-inflammatory and anticancer potential of the bioactive fraction extracted from the flower of *L. philadelphicum* (Red Lily) and characterization of these bioactive compounds. The antimicrobial activity was tested against nine different Gram-positive and Gram-negative bacterial strains. The minimum inhibitory concentration (MIC) values of methanolic extract of *L. philadelphicum* flower against *Acinetobacter bouvetii*, *Achromobacter xylosoxidans*, *Bacillus subtilis* MTCC 121, *Candida albicans* MTCC 183, *Klebsiella pneumoniae* MTCC 3384, and *Salmonella typhi* MTCC 537 were 25, 50, 12.5, 50, 100 and 50 μg mL^{-1}, respectively. The phytochemical analysis of the extract reveals the presence of phenols, flavonoids, tannins, terpenoids, glycosides, coumarins, and quinones. The cytotoxicity of the partially purified compound against the HepG2 cell line in MTT assay demonstrates up to 90% cell viability with a bioactive compound concentration of 50 μg/ml. However, with the increase in bioactive compound concentration up to 1000 μg/ml results into nearly 80% cell viability, just a minor decline in cell viability suggests the importance of bioactive compounds for suitable therapeutic applications. Spectroscopic studies of the bioactive compound by UV-Visible spectroscopy, FT-Infra Red spectroscopy, Gas Chromatography-Mass Spectrometry (GCMS) as well as its phytochemical analysis suggests the presence of terpenoids moiety, responsible for the antimicrobial property of *L. philadelphicum* flower.

Keywords: Antimicrobial activity; Characterization; GC-MS analysis; FTIR analysis; Red lily; Secondary metabolites.

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1. Introduction

Ayurveda is an ancient Indian script comprising valuable information about herbal remedies, indicating the potential of plants to cure various diseases in human beings. Modern research has explained that plants contain diverse secondary metabolites, which are often not involved directly in the metabolism but somehow play a major role in plant defense system [1]. Plant-derived secondary metabolites, such as terpenoids, phenols, flavonoids, and coumarins are reported to possess antimicrobial properties and therefore reveal that Mother Nature is a potential source of therapeutic agents [2]. Over the past century, natural products such as vincristine from *Vinca rosea* and morphine from *Papaver somniferum* have been developed as best-selling medicines [3]. India has a great treasure of medicinal plants due to which it is one of the richest nations in terms of a vast collection of genetic resources of medicinal plants in the world [4].

The members of the Liliaceae family have been found to contain phytochemicals such as alkaloids, steroidal saponins, vitamins, and fatty acids, which are responsible for their biological activity [5]. Flowers of various species of lily have been reported to possess broad-spectrum antimicrobial activity [5,6] *L. longiforum*, has been studied and used as an anti-inflammatory agent for the treatment of bronchitis and blood clotting during the surgical procedures[70. Lipid peroxidation and cholesterol oxidase enzyme inhibitor assays are used to determine the bioactive compounds in *L. longiforum* flower, which in turn shed light on its anecdotal medicinal use [8]. The present work was aimed to evaluate the antimicrobial, anti-inflammatory, and anticancer potential of the bioactive fractions extracted from the flower of *L. philadelphicum* and to analyze the phyto-chemical and spectroscopic studies. Evaluation of antimicrobial properties of the extracted compound from *L. philadelphicum* flower is expected to provide new dimensions in the treatment of infectious diseases.

2. Materials and Methods

2.1. Source of L. philadelphicum flowers and microbial cultures

L. philadelphicum flowers used in this study were collected from the campus of the Institute of Engineering and Technology, Lucknow, Uttar Pradesh, India. Microbial cultures namely *Achromobacter xylosoxidans* and *Acinetobacter bouvetii* were obtained from the culture repository of the institute while other cultures such as *Bacillus pumilus* MTCC 1607, *Bacillus subtilis* MTCC 121, *Candida albicans* MTCC183, *Escherichia coli* MTCC 1304, *Klebsiella pneumoniae* MTCC 3384, *Salmonella typhi* MTCC 537, *Staphylococcus aureus* MTCC 96 and *C.albicans* were procured from Microbial Type Culture Collection (MTCC), Chandigarh (Punjab), India.

2.2. Purification and characterization of bioactive compounds from the red lily flower

L. philadelphicum flowers collected from the college campus (Institute of Engineering and Technology, Lucknow, Uttar Pradesh, India) were used in this study. Flowers were dried under shade at 30-40 °C and subsequently powdered. The crude powder was extracted with various solvents *viz.* hexane, chloroform, methanol, and water in a ratio of 1:10 (crude powder: solvent ratio) at 28 °C. The extracts were filtered with Whatman filter paper, concentrated under vacuum, and stored at 4°C until further use. The extraction yields of different solvents were determined using the following formula.

$$\text{Extract yield } = \frac{W1}{W2} x\ 100$$

Where,
W1 is the net weight of the flower powder in gm after extraction, and

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W2 is the total weight of the flower powder in gm taken for extraction.

2.3 Evaluating antimicrobial activity of the red lily flower extract

The antimicrobial activity of the crude metahnolic extract of the lily flower was determined using agar well diffusion method against *A. xylosoxidans*, *A. bouvetii*, *B. pumilus* MTCC 1607, *B. subtilis* MTCC 121, *C. albicans* MTCC183, *E. coli* MTCC 1304, *K. pneumoniae* MTCC 3384, *S. typhi* MTCC 537 and *S. aureus* MTCC 96. Twenty-four h old bacterial and fungal test cultures were grown on nutrient agar (NA) (Hi-Media, Mumbai, India) and potato dextrose agar (PDA) (Hi-Media, Mumbai, India), respectively the wells of 9 mm diameter were bored and filled with 200 µL of the solvent extracts of the red lily flower and the culture plates were incubated at 37 °C for 24 h for bacterial and 72 h for fungus. After the incubation plates were observed for the inhibition of growth of test organisms and the diameter of the zone of inhibition from each plate was measured [9].

2.3.1 Phytochemical screening

The methanolic extract of *L. philadelphicum* flower was screened to examine the presence of chemical groups and active compounds such as carbohydrates, saponins, phenols and tannins, coumarins, flavonoids, amino acids, glycosides, terpenoids, and quinones.

To detect the presence of carbohydrates, the flower extract was dissolved in 5 mL distilled water and filtered. The filtrate was hydrolyzed with dilute HCl and further neutralized with alkali and subsequently heated with Fehling's solution A and B and observed for the formation of a red precipitate of reducing sugars [10]. The examination of saponin was performed by the foam test. The flower extract (0.5 g) was vigorously mixed with 2 mL of water and observed for the foam formation for more than 10 min as an indication of the presence of saponin [10]. The presence of phenols and tannins were detected by performing the ferric chloride test. Ferric chloride (0.5%) solution was added drop by drop to 2 mL of flower extract and observed for the formation of a bluish-black precipitate of phenols and tannins [10]. About 0.5 g of the moistened flower extract was taken into the test-tube. The mouth of the test-tube was covered with the filter paper treated with 1 N NaOH solution. The treated test-tube was placed in boiling water for a few minutes and examined for the formation of yellow color as an indication of the presence of coumarins [11]. To test the presence of flavonoids in the flower extract, a 10% lead acetate solution was added in the extract. The formation of yellow precipitate confirmed the presence of flavonoids [10]. The presence of amino acids in *L. philadelphicum* flowers was checked by employing a ninhydrin test. Few drops of ninhydrin solution were added to the flower extract and the appearance of blue color indicated the presence of amino acid [12]. For the identification of glycosides, 1 ml of glacial acetic acid, few drops of ferric chloride solution, and concentrated H_2SO_4 (mixed slowly through the sides of the test-tube) were added to the flower extract and observed for the appearance of a reddish-brown ring of de-oxy sugars at the junction of the liquids [10]. For terpenoids identification in flower extract, 2 ml of chloroform was added in 5 ml of the flower extract and thereafter 3 ml of concentrated H_2SO_4 was added slowly and observed for the appearance of the reddish-brown color of terpenoids [10]. The flower extract was treated with a few drops of concentrated H_2SO_4 and observed for the formation of yellow color as an indication of the presence of quinones compound(s) [10]. The partial purification of the crude extract of *L. philadelphicum* flower was performed by column chromatography using silica gel (mesh size 230-400) as a matrix. The column was eluted successively with an increasing gradient of methanol and chloroform. Further, the fractions were collected and examined by thin-layer chromatography (TLC) using a silica plate (TLC silica gel 60 F254) with methanol: chloroform (0.2% to 5%) as the mobile phase. TLC was developed in the iodine chamber and the fractions having the same retention factor (R_f) values were pooled together and subjected to the bioassay [10].

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2.4. Characterizing the bioactive fractions

The chemical characterization of the active fraction was done with the help of UV-Visible Spectroscopy (Perkin Elmer UV WinLab 5.2.0.0646/ Lambda 25 spectrophotometer), Fourier Transform Infrared Spectroscopy IR (FT-IR Perkin Elmer Model RX-1 spectrometer), and Gas Chromatography-Mass Spectroscopy (TSQ Quantum XLS).

2.5. Estimating minimum inhibitory concentration (MIC) of the partially purified compound

The MIC values of the partially purified compound were checked against Gram-positive and Gram-negative bacterial strains and fungus with erythromycin (E15) as a standard antibacterial drug according to standard protocol [13]. A 24 h old culture of each bacterial test strain ($5x10^{-5}$ cells mL^{-1}) was grown in on NB and 200 µg/ml of the compound was taken as an initial concentration in the first test-tube and was serially diluted. NB but with erythromycin served as a negative and positive control, respectively. Another control, i.e. pure solvent (DMSO) only was also included to observe the effect of the solvent on microbial growth. All the tubes were incubated at 28 °C overnight and thereafter the bacterial growth was observed [13].

2.5.1 Analyzing cytotoxicity and MTT

In vitro cytotoxicity of the compound against HepG2 cell lines was analyzed by MTT assay as described by Mosmann [14] with minor modifications. A 100 µL of HepG2 cells suspension in 96 well microtitre plate was incubated overnight at 37 °C in a CO_2 incubator. After 24 h, the medium was replaced by 100 µL of the fresh medium and treated with varying concentrations of the flower extract (0 - 1000 µg mL^{-1}). The microtitre plate was re-incubated at 37 °C under 5% CO_2 in the air for 24 h. The wells without flower extract served as a positive control. Further, MTT at 0.5 mg mL^{-1} concentration was added to the cell culture and the plate was further incubated at 37 °C for 4 h. After the incubation, culture supernatant was removed and the cell layer was dissolved in DMSO (200 µl) and analyzed in a microplate reader (BioTek Instruments Inc, Vermont, USA) at the test wavelength of 550 nm and the reference wavelength of 660 nm.

3. Results and Discussion

3.1. Antimicrobial and phytochemical screening of solvent extracts

The investigation on the reddish-orange bioactive compound extracted from *L. philadelphicum* reveals the presence of polar compounds. The methanolic and aqueous extract has greater extraction yields as 23.12 and 23.54%, respectively, among four solvents used during the extraction process. The yields obtained from chloroform and hexane extracts were 2.25 and 10.29%, respectively. The flower extract exhibited antimicrobial activity against *S. typhi* MTCC 537, *A. bouvetii*, *B. subtilis* MTCC 121, *A. xylosoxidans K. pnemoniae* MTCC 3384, and *C. albicans* MTCC 183. However, the methanolic extract was not effective against *S. aureus* MTCC 96, *B. pumilus* MTCC 1607, and *E. coli* MTCC 1304. The aqueous extract of *L. philadelphicum* flower exhibited antimicrobial activity against *B. subtilis*, *B. pumilus*, *K. pneumoniae*, *A. bouvetti,* and *C.albicans*. However, chloroform and hexane extract was found slightly effective only against *K. pneumoniae* MTCC 3384 and *A.xylosoxidans.*

In developing countries like India, various infectious diseases are still creating challenges in the healthcare sector. To counteract against such challenges, a variety of antimicrobials have been discovered to fight against the pathogens responsible for various diseases. These antimicrobials can be obtained as secondary metabolites from microbes, animals, or plants, or maybe synthesized chemically [15,16]. Due to the occurrence of wide-spectrum and multiple drug resistance (MDR) in

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pathogens towards existing antibiotics besides the unpleasant side effects of the currently used antibiotics and synthetic drugs, investigation of other sources like medicinal plants, for their antimicrobial properties are gaining importance [17]. The phytochemical analysis of *L. philadelphicum* flower revealed the presence of flavonoids, tannins, glycosides, phenols, coumarins, and terpenoids (Table 1).

Table 1. Phytochemical screening of various extracts of red lily flower

Compounds	Extract			
	Aqueous	Chloroform	Methanol	Hexane
Saponins	-	-	-	-
Phenols	+	+	+	-
Glycosides	+	+	+	+
Flavonoids	+	+	+	+
Carbohydrates	-	-	-	-
Proteins	-	-	-	-
Coumarins	+	+	+	-
Quinones	+	-	+	-
Tannins	+	-	+	-
Terpenoids	+	-	+	-

'+' Present; '-' Absent

Several bioactive constituents have been reported from plants like *Azardirachta* indica [18], *Senna alata* [18], and *Terminalia bellerica* [20]. Although, the researchers are more focused on the studies on biological activities of higher plants, however, relatively fewer studies are reported dealing with the isolation, purification, and characterization of biologically active compounds from flowers. In efforts for the search of potential antimicrobial compounds, Dontha et al [21] reported the isolation of active constituents of *Ixora Javanica* D.C flower extract and it's phytochemical characterization. Similarly, in our study, the flower of *L. philadelphicum* has been investigated and the reddish-orange bioactive compound has been isolated from the methanolic extract. The flower extract was found active against some Gram-negative/-positive bacteria and *Candida albicans.* Soliman et al [16] reported the antifungal activity of a range of plants and suggested that they can be used as anti-candida agents after some more *in vivo* investigations and studies regarding the use of nano-structured lipid system [22].

3.2. Purification and chemical characterization of bioactive compound

The flower's crude metahnolic extract purified on silica column showed the presence of forty-eight fractions, these fractions were separately collected their Rf values was determined and all the fractions were subjected to antimicrobial bioassay. The initial fractions obtained from the silica column were more active against all the test organisms. However, fractions eluted later were active against one or two microbes only **(Figure 1).** The most active fraction was selected and subjected to partial characterization by UV visible, FTIR, and GC-MS spectrum. The active fraction absorbed in the range of 290 to 360 nm suggesting the presence of a double bond in the conjugation (**Figure 2**).

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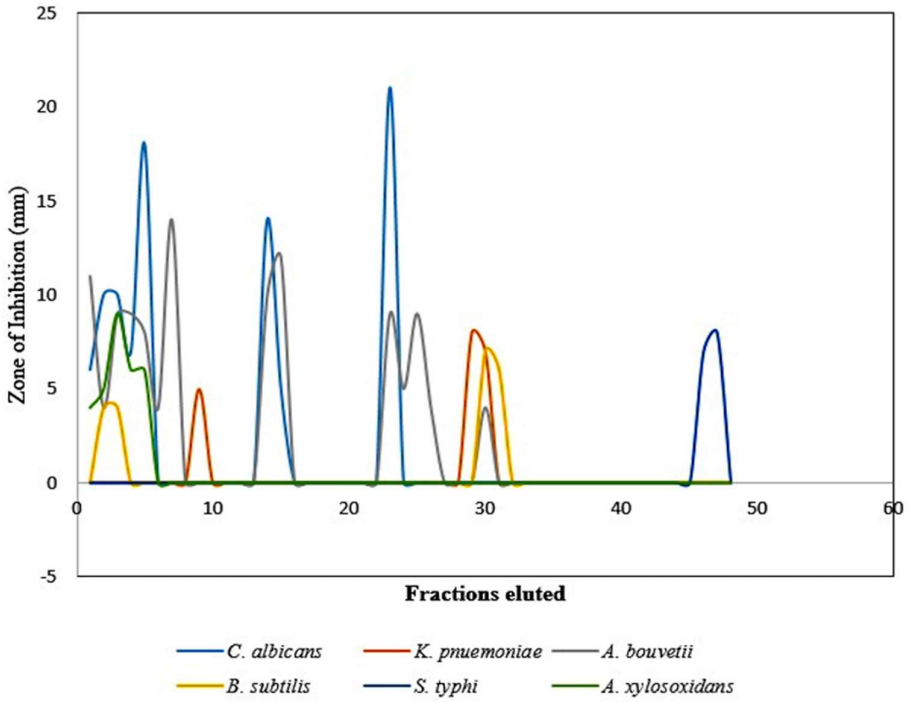

Figure 1: Activity profile of the column passed fractions

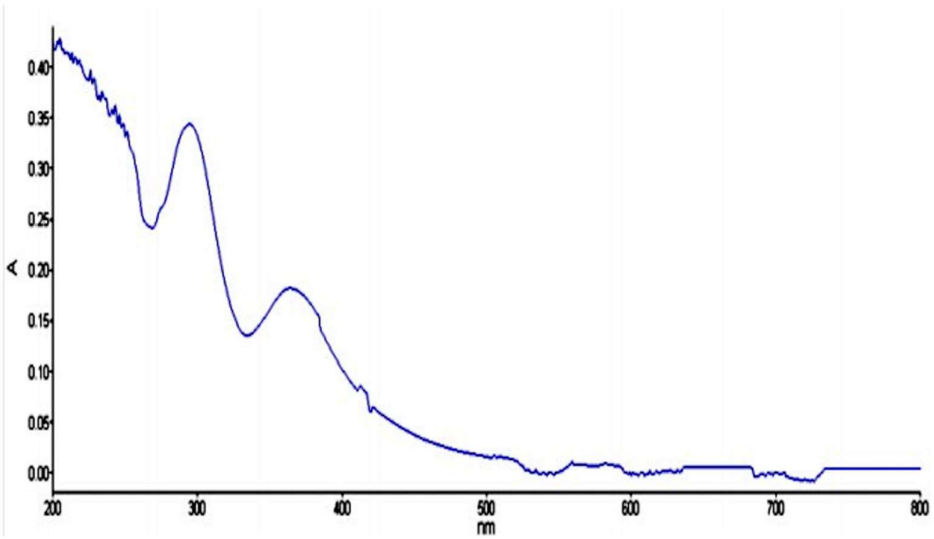

Figure 2: UV profile of the active compound in methanol

The presence of strong absorption bands in the ultraviolet (UV)/visual (VIS) absorption spectrum is possibly due to p→p* or n→p* transitions and the consecutive bands suggest the presence of conjugation in the structure [23]. IR spectrum, explains different types of bonds i.e. single, double or triple among carbon, hydrogen, nitrogen, and oxygen atoms, which have diverse vibrational frequencies, can be identified [24].

FTIR spectrum of the samples exhibits bands at 2994.75 and 2911.83 cm^{-1}, which confirms the CH stretching (**Figure 3**). The band at 1435.83 cm^{-1} indicates the aromatic or heteroatomic C-C

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stretching vibrations. The absorption band at 1309.05 cm^{-1} corresponds to C=O moiety in the compound. The frequencies were identified at 951.52, 696.04, and 666.66 cm^{-1}corresponds to =C-H bending vibrations.

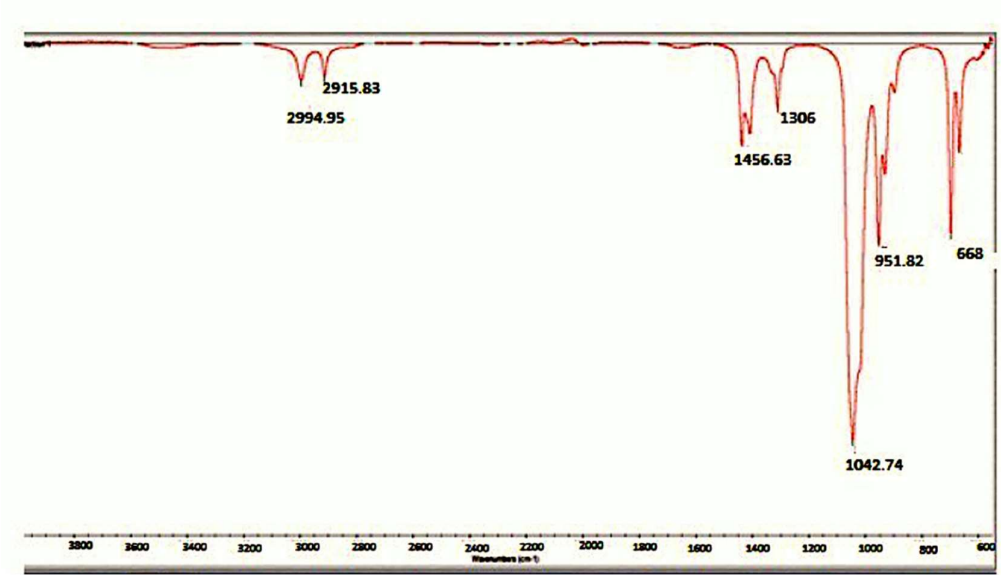

Figure 3. FTIR spectrum of the active compound in methanol

GC-MS spectrum shows nine major ideal peaks with different retention times as 13.30, 18.93, 24.97, 28.04, 30.91, 33.46, 36.02, 40.77 and 44.62 min (**Figure 4**).

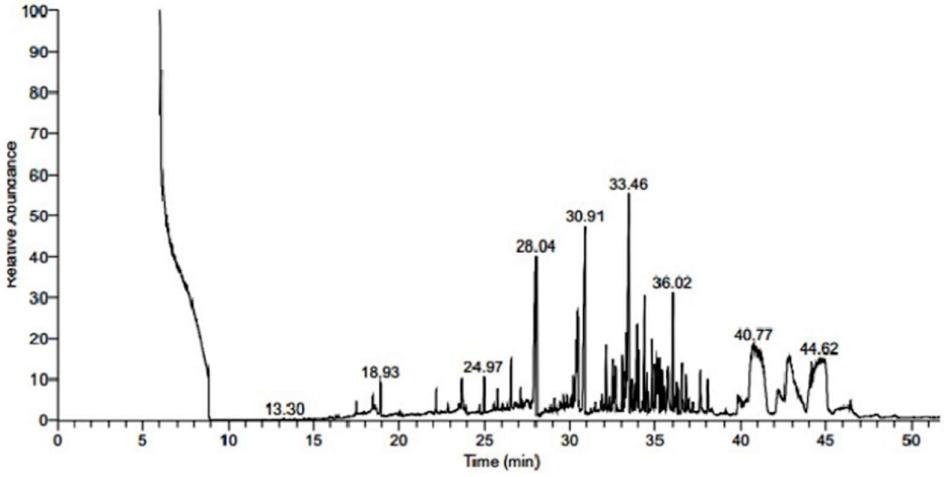

Figure 4: GC-MS profile of the active fraction

Further, GC-MS of the sample was recorded on TSQ Quantum XLS for the identification of the bioactive component(s) of the flower. The generated spectrum was processed for major ideal peaks found during the analysis and compared with the mass spectrum from the library standards available in the database (NIST library). The GC-MS spectrum shows nine peaks at different retention times (RT) with different probability factors (PF), which in turn reflects the presence of nine different

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compounds. These compounds are chloculol (RT 13.30 min; PF 68.43; m/z 205.1); (+ -)-5- Hydroxy-6-(1-hydroxy ethyl)-2, 7-dimethooxynapthoquinone (RT 18.93 min; PF 2.93; m/z 263.2); 1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester (RT 24.93 min; PF 30.42; m/z149.0); 12-methoxy-2-trimethylsilyloxy-19-nor-5ápodocarpa-1,3,8,11,13-pentaene (RT 28.04 min; PF 50.12; m/z314.1); 3-Acetoxy-7á-(acetylthio)-17á pregna-3,5-diene-21,17-carolactone (RT 30.91 min; PF 26.29; m/z 341.3); 2-(Dimethyl phenylsiyl) hepta-1,5,diene-4-one (RT 33.46 min; PF 19.82; m/z 135.1); 3-[(t-butyl diphenylsilyl) oxy]-4-benzyloxy-6-acetoxy-6-vinyl-1-bromo-1-cyclohexene (RT 36.02 min; PF 43.88; m/z 399.4); 1, 4, 5, 7, tetrahrdoxy-2-methylanthaquinone (RT 40.77 min; PF 46.34; m/z 207.2) and silane (RT 44.62 min; PF 56.42; m/z 129.0) [19].

The cytotoxicity of the active fraction determined by MTT assay using the HepG2 cell line in this study concludes that ~90% of cells survived up to 50 µg/ml concentration of the bioactive compound. The active fraction may contain terpenoids as evident by the spectroscopic and phytochemical analysis. The terpenoids in the active fractions might be responsible for the antimicrobial properties of *L. philadelphicum* flower. To the best of our knowledge, this is the very first study deciphering the antimicrobial activity of *L. philadelphicum* flower [24].

Estimating minimum inhibitory concentration (MIC) and cytotoxicity of the active fraction

The MIC of the partially purified compound against *Candida albicans* MTCC 183, *Achromobacter xylosoxidans*, and *Salmonella typhi* MTCC 537 was 50 µg/ml. The purified compound is less active (shows MIC 100 µg mL^{-1}) against *Klebsiella pneumoniae* MTCC 3384. However, it is found more effective against *Acinetobacter bouvetii* and *Bacillus subtilis* MTCC 121 with MIC values of 25 and 12.5 µg/m, respectively. Further, the cytotoxicity analysis of the bioactive compound on the HepG2 cell line using MTT assay demonstrates approximately 9.7% cell inhibition at 50 µg/m concentration and approximately 20% cell inhibition at 1000 µg/ml concentration (**Figure 5**).

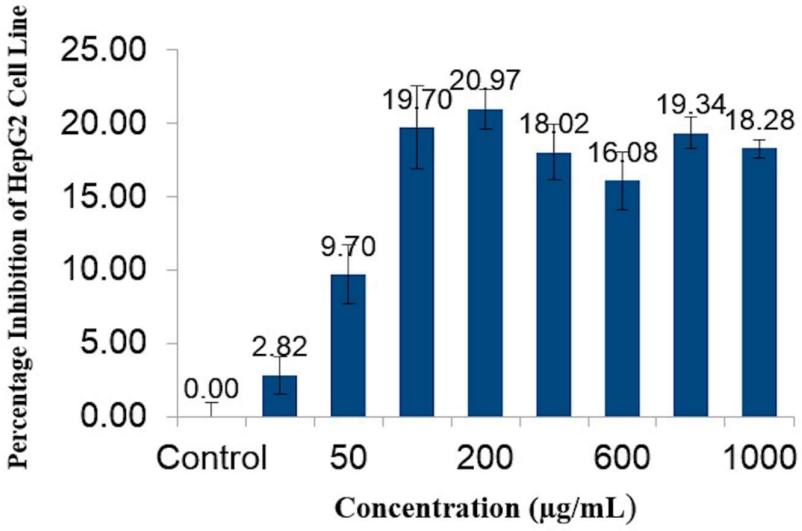

Figure 5. Cytotoxic activity of active fraction against HepG2 cell line

The phytochemical analysis of *L. philadelphicum* flower is in line with earlier findings, where it has been reported that carbonyl compounds such as terpenoids, especially monoterpenes [C10], and sesquiterpenes [C15], alcohols, aldehydes, acyclic esters or lactones, and exceptionally nitrogen and sulfur-containing compound, coumarins, and homologs of phenylpropanoids, exhibit a wide

spectrum of biological activity [25]. The active fraction may contain terpenoids as evident by the spectroscopic and phytochemical analysis. The terpenoids in the active fractions might be responsible for the antimicrobial properties of *L. philadelphicum* flower. To the best of our knowledge, this is the very first study deciphering the antimicrobial activity of *L. philadelphicum* flower.

Conclusion

The potent and broad-spectrum antimicrobial properties of *L. philadelphicum* (red lily) flower suggests its therapeutic potential against Gram-positive and Gram-negative pathogens. The phytochemical analyses reveal that the polar organic solvents are more efficient in the extraction process, thus suggests the polar nature of the metabolites present in the flower. Further, the cytotoxicity assay concludes that approximately 90% of cells are viable up to 50 µg/ml concentration of the partially purified compound from *L. philadelphicum* flower. In conclusion, the antimicrobial activity of *L. philadelphicum* flower along with cytotoxicity results suggests the future application of this flower for antimicrobial purposes in the treatment of various infectious diseases. Further research in the direction of complete characterization of the active compound and *in vivo* mechanistic studies in animal models may provide better insight into the understanding of the identification and development of suitable bioactive agents to treat various infectious diseases.

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Authors contribution : Methodology, Investigation ; SS, Formal analysis; VS, Conceptualization and Supervision BNM and NM, Reviewing and Editing RZS, SH and DE

Conflict of Interest Statement

The authors declare no conflict of interest exists.

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