Berberine from Argemone mexicana L exhibits a broadspectrum antibacterial activity

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The Argemone mexicana L, commonly found on desolate land in the Marathwada region of Maharashtra state, India, has been used for treating oral cavity infections. We sought to investigate the antimicrobial potential of A. mexicana L. In this study, cold aqueous and methanolic extracts were prepared from the A. mexicana L leaves. These extracts were tested for their antibacterial activities against selected bacterial isolates. The antibacterial activity and MICs were tested using the agar well diffusion method and broth dilution method, respectively. The cold aqueous and methanolic extracts of A. mexicana L leaves inhibited growth of clinical isolates of Staphylococcus aureus, Bacillus cereus. Escherichia coli and Pseudomonas aeruginosa. The antibacterial potentiality of A. mexicana L extracts was compared with Streptomycin – the reference antibiotic used in this study. The active ingredient of antibacterial potentiality within the A. mexicana L extract was purified and characterized by TLC, HPLC and NMR analysis. Structural elucidation of Berberine and its bioactivity both, from the A. mexicana L and commercial preparation, is investigated.

Key words: antibacterial potentiality, Argemone mexicana, aqueous, methanolic extract, HPLC, NMR and phytochemical analysis

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INTRODUCTION

About 80% of the population in developing countries relies for its primary health care needs on traditional and/or alternative medicine which is based on medicinal plants (Newman & Cragg, 2007). It is estimated that about 87% of all categorized human diseases, including microbial infections, cancer, metabolic disorders and immunological disorders, have been treated with natural products and alternative medicines (Uddin et al., 2011). Commonly used medicinal plants and vegetables not only possess the essential nutrients but are also reported to contain secondary metabolites, such as alkaloids, flavonoids, glycosides, terpenoids and phenolics (Cai et al., 2004; Abdelwahab et al., 2010). Currently, there is a great number of antibiotics available to treat microbial infections, however, it is well documented that many infectious agents have attained resistance to several of these antibiotics (Roux et al., 2012; Kapoor et al., 2017). This has posed a great difficulty in treating microbial infections. An introduction of fixed drug combinations could override this difficulty in a few cases (McGettigan et al., 2015). However, for many microbial infections, in particular bacterial ones, even a fixed drug combination approach has been also found to be ineffective. For over one and a half decades no new antibiotics with the ability to escape the microbial drug resistance phenomenon have been added (Sorg et al., 2016). The Argemone mexicana L. (Papaveraceae), commonly known as prickly poppy, has been used as a medicinal plant in Mexico, Nigeria and tropical America (Rosas-Pinon et al., 2012; Gupta et al., 2015). It is used to treat different ailments, given its antimicrobial, antiparasitic, antimalarial, pesticide, cytotoxic and neurological properties (Graz et al., 2010; Rubio-Pina & Vazquez-Flota, 2013). Fresh yellow, milky seed extract containing protein-dissolving substances is effective in the treatment of warts, cold sores, skin diseases, itches and jaundice (Chopra et al., 1986). Previous studies by (Bhattacharjee et al., 2006; Singh et al., 2009; Osho & Afetonji, 2010; Alagesaabopati & Kalaiselvi, 2012 and Sahu et al., 2012) reported antibacterial activity in root, stem, leaves, seed and essential oil extracts of A. mexicana L. In-vitro antibacterial activity of the stem of A. mexicana L and wild medicinal plants were also evaluated by (Rahman et al., 2009 and Saranya et al., 2012). Recently, antifungal (Doss et al., 2012 and More & Kharat, 2016) and anticancer potentiality of A. mexicana L has been reported (More & Kharat, 2016). Despite the number of antibacterial potentiality reports on A. mexicana L, it is unclear which of the phytochemicals are responsible for this potentiality. In this study, we aimed to explore the existence of antibacterial potential for A. mexicana against pathogenic bacteria. The second objective of this study included isolation, purification and characterization of the phytochemicals conferring antibacterial potentiality to A. mexicana L. Cold aqueous and methanolic extracts prepared from A. mexicana leaves were processed for determination of antibacterial potential against pathogenic isolates: Gram positive, i.e. Staphylococcus aureus and Bacillus cereus, and Gram negative, i.e. Escherichia coli and Pseudomonas aeruginosa. The results obtained on the antibacterial potential and analytical studies on the characterization of the active ingredient and functional confirmation on antibacterial potentiality are discussed.

MATERIALS AND METHODS

Bacterial culture maintenance. The bacteria used in this study included Gram positive bacteria: Staphylococcus aureus (Amox³ & Amp³) and Bacillus cereus, and Gram negative bacteria: Escherichia coli (Amp³ & Chl³) and Pseudomonas aeruginosa (Pen³ & Amp³). They were maintained on nutrient agar (NA) slants at 4°C (HiMedia India). The bioassay of bacterial suspension was obtained by inocula-
tion using nutrient broth for 24 h, followed by ten-fold serial dilution in PBS pH 7.2 to obtain CFU/mL = 10³.

**The A. mexicana plantparts.** The plant material was verified and authenticated with the Herbarium Center, Department of Botany, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad, India. It was confirmed as A. mexicana L., a member of the Papaveraceae family and is commonly referred to as Swarnakshiri, Bilayat and Pivla Dhotra. The allotted accession number is 0609. Leaves of A. mexicana were collected and rinsed with sterile double distilled water, disinfected with 70% alcohol and then dried on paper towel at room temperature. After drying, the plant materials were ground in a laboratory grinding machine.

**Methanolic extracts.** The methanolic extracts were prepared as described in More & Kharat (2016). Briefly, in a tightly sealed container at room temperature, 50 g of the grounded plant material were extracted with 150 mL of methanol. The extract was protected from light and kept overnight on a rotary shaker (Remi, Elektrotechnik, Ltd., Mumbai, India). The extract was filtered through a five layered sterile muslin cloth. The procedure was repeated three times to obtain clear and colorless filtrate. The methanol from the filtrate was removed by rotary evaporation (Rotary Evaporator, EJER tech, Hangzhou, Zhejiang, China). Extract was stored at 16°C overnight, and subsequently freeze-dried at -60°C in a 20 mL vacuum for 24 h. The extract was then sterilized with UV and stored in an airtight container at 4°C until further use.

**Aqueous extracts.** Exactly 50 g of grounded plant material were extracted with 150 mL sterile double distilled water for 24 h, as in the case of methanol. The mixture was filtered through a sterile five-layered muslin cloth and centrifuged at 5000 rpm, and the supernatant was concentrated with the rotary evaporator (EJER tech, Hangzhou, Zhejiang, China). The concentrated extract, free from liquid, was then UV sterilized and stored at 4°C until further use. At the time of experiment, the dry mass was resuspended to achieve 2 mg/ml concentration.

**Antibacterial potentiality test.** The antibacterial potentiality of A. mexicana was tested with minor modifications byan agar well diffusion method described by (Boyanoval et al., 2005). An inoculum of 10⁶ CFU/mL was adjusted to molten Muller Hinton agar and poured into plates that were previously pre-sterilized petri-plates. Upon solidification, wells were punched with a sterile cork borer (Scientific laboratory, New Delhi, India). Each well was filled with 40 µL of the extract (20 µL). As an antibacterial standard, streptomycin (40 µg) was used for comparison. Plates were then incubated at 37°C for 24 h for the detection of inhibitory zones. The experiments were repeated three times; the average and standard deviations (S.D.) were then calculated. Antibacterial activity was evaluated by measuring the diameter of growth inhibition zone around the well.

**Phytochemical analysis of the A. mexicana leaf extracts.** Qualitative and quantitative estimates for various phytochemicals of A. mexicana extracts were performed. Existence of phytochemicals was confirmed by performing various biochemical tests, namely the Molish’s test for carbohydrates, ninhydrin test for proteins, FeCl₃ test for tannins, foam test for saponins, fluorescence test for coumarins, Fehling’s test for glycosides, Shinoda test for flavonoids, Liebermann Burchard test for steroids and terpenoids, as well as Dragendorff test for alkaloids, as described in Rajpal (2005). For the estimation of phytochemicals, 1 g of powder dissolved in 20 mL of water was incubated in a water bath incubator at 65°C for 15 min and filtered through five layered sterile muslin cloth. The filtrate obtained was used in the analysis of carbohydrates, saponins, proteins and tannins.

**Minimum inhibitory concentration (MIC) of the leaf methanolic extracts of A. mexicana.** The MIC was calculated as the lowest concentration that inhibits growth of the tested organism. Minimum inhibitory concentration (MIC) was determined by agar well diffusion method (Boyanoval et al., 2005) and standard broth dilution techniques (Washington & Sutter 1980). On each Muller Hinton agar plates, 8 mm wells were punched with a sterile cork borer. Sample extracts were prepared at a concentration of 10 mg/ml, 20 mg/ml, 30 mg/ml and upto 100 mg/ml. The plates were then incubated at 37°C for 18 to 24 h. The inhibitory zone which appeared around the well was recorded. In the case of broth dilution, bacteria were inoculated in Muller Hinton broth supplemented with 200 mg/ml, 400 mg/ml, 800 mg/ml, and 1600 mg/ml of the leaf methanolic extracts of A. mexicana. Tubes were incubated at 37°C for 24 h, the lowest concentration inhibiting bacterial growth was expressed as MIC in mg/ml.

**Thin layer chromatography for crude extracts and berberine chloride.** The methanolic extracts of A. mexicana leaves and berberine (Sigma-Aldrich) were prepared by dissolving in methanol (mg/ml) and collected in two test tubes. The TLC plates, 15 cm×10 cm×0.3 cm (length×width×thickness) were overlaid with a slurry of silica gel with calcium sulphate as the binder. An aliquot of 20 µl sample was loaded onto the thin layer chromatography plate and put in a buffer tank containing Hexane: chloroform: methanol (4:4:2, V/V/V); thereusulting chromatogram bands were visualized by iodine vaporization.

**Column chromatography conditions.** A 1.5 cm×30 cm (diameter×height) column was packed with 15 g of silica powder made in the n-Hexane solvent. The extract was then loaded onto the top of the column and saturated for 3 h. The elution was done by using 20 different mobile phases (Table 3) shifting from non-polar to polar in an increasing order. These fractions, along with the berberine, were then used for the TLC analysis. The Rf values for each fraction were recorded and compared with berberine. Fraction 12 indicated similar Rf value with berberine. Hereafter, fraction 12 was utilized for further ultra-purification with HPLC and deciphering structure with 1H NMR.

**High Performance Liquid Chromatography conditions.** Analysis was performed using a high performance liquid chromatography system (Shimadzu’s Prominence HPLC class LC-20AD, Japan), with a Prominence Pump, High precision dual plunger design and forced check valve design for excellent solvent delivery control (Schevenning et al., 2008). A reverse-phase C18 column (length 250 mm, dia – 4.6 mm, particle size 5 µm, pore size 100 Å, Material: Silica, Spherical Fully Porous Ultrapure) was used (Synchroms, ThermoFisher Scientific, Inc, USA). All analyses were performed at room temperature with a mobile phase of methanol and acetonitrile (60:40), an injection volume of 10 µl, and a flow rate of 1.0 mL/min. The column effluent was monitored at 214 nm with an L-2400 series multi-wavelength UV Detector. Fractions were collected for further analysis of antibacterial activity and structure determination on the basis of their RT.

**NMR spectroscopy conditions.** NMR studies were conducted to predict the structure of the chemical compound present in the HPLC purified fraction. The analysis was performed with the Advance – III 400 MHz
Fourier Transform Digital NMR Spectrometer (Bruker Biospin, Switzerland) 1H in DMSO-D6. 1H NMR spectra of HPLC Peak 2 and standard berberine chloride were recorded. Chemical shifts were recorded as a delta value. For graphical analysis, amestrenova software was used. The software enabled identification of the compound present in the HPLC purified fraction.

**RESULTS**

**Antibacterial potential of** *A. mexicana*

One of the aims of this study was to address whether or not *A. mexicana* has antibacterial potentiality against clinical isolates. The methanolic and cold aqueous extracts, while empty bars denote antibacterial activity of streptomycin. Error bars shown on each histogram indicate standard deviation.

### Table 1. Determination of MIC for the *A. mexicana* methanolic and aqueous extracts.

<table>
<thead>
<tr>
<th>Sr. No</th>
<th>extract in μg/well</th>
<th>Zone of Inhibition (in mm)</th>
<th>B. cereus</th>
<th>S. aureus</th>
<th>P. aeruginosa</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Methanolic</td>
<td>Aqueous</td>
<td>Methanolic</td>
<td>Aqueous</td>
<td>Methanolic</td>
</tr>
<tr>
<td>01</td>
<td>200</td>
<td>10±1/1</td>
<td>10±1/1</td>
<td>10±1/2</td>
<td>10±1/2</td>
<td>10±1/1</td>
</tr>
<tr>
<td>02</td>
<td>400</td>
<td>15±2/1</td>
<td>16±1/1</td>
<td>14±1/1</td>
<td>13±1/2</td>
<td>16±1/1</td>
</tr>
<tr>
<td>03</td>
<td>600</td>
<td>17±1/1</td>
<td>20±1/2</td>
<td>17±1/1</td>
<td>17±1/1</td>
<td>18±1/1</td>
</tr>
<tr>
<td>04</td>
<td>800</td>
<td>21±1/1</td>
<td>20±1/2</td>
<td>22±1/1</td>
<td>22±1/1</td>
<td>22±1/1</td>
</tr>
<tr>
<td>05</td>
<td>1000</td>
<td>21±1/1</td>
<td>20±1/2</td>
<td>22±1/1</td>
<td>22±1/1</td>
<td>22±1/1</td>
</tr>
</tbody>
</table>

Note: Values for inhibitory zones were recorded from at least three experiments, ±/+ denotes Standard deviation.

### Table 2. Detection of phytochemicals from the methanolic extracts of *A. mexicana* leaves

<table>
<thead>
<tr>
<th>Phytochemical compounds</th>
<th>Leaf Extract</th>
<th>Phytochemical compounds</th>
<th>Leaf Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate</td>
<td>Present</td>
<td>Glycoside</td>
<td>Present</td>
</tr>
<tr>
<td>Saponin</td>
<td>Absent</td>
<td>Flavonoid</td>
<td>Absent</td>
</tr>
<tr>
<td>Protein</td>
<td>Absent</td>
<td>Phytosterol</td>
<td>Absent</td>
</tr>
<tr>
<td>Split amino acids</td>
<td>Absent</td>
<td>Phenolics</td>
<td>Absent</td>
</tr>
<tr>
<td>Tannin</td>
<td>Present</td>
<td>Alkaloid</td>
<td>Present</td>
</tr>
<tr>
<td>Coumarins</td>
<td>Present</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The antibacterial potential identified in *A. mexicana* leaf extract was confirmed with agar well diffusion assay as described above. The HPLC purified extract, along with berberine and a comparison standard antibiotic streptomycin, were used for the antibacterial assay. The inhibitory zones against test organisms were analyzed.

**Figure 1A.** Antibacterial activity of methanolic and aqueous extract against four tested bacteria:
The X-axis shows clinical isolates used in this study; Y-axis shows zone of inhibition represented by bars: (1) *B. cereus*; (2) *S. aureus*; (3) *P. aeruginosa*; and (4) *E. coli*. Antibacterial activity within methanolic extracts of leaves (black bars), aqueous (gray bars) for *A. mexicana* extracts, while empty bars denote antibacterial activity of streptomycin. Error bars shown on each histogram indicate standard deviation.

**Figure 1B.** Functional demonstration of antibacterial potency of purified berberine:
Zone of inhibition in mm against different Gram positive and Gram negative bacteria, standard berberine chloride, HPLC purified fraction and streptomycin. The black bars indicate berberine, gray bars indicate HPLC peak 2, while the empty bars denote streptomycin mediated antibacterial activity.
Table 3. Mobile Phase used for column chromatography

<table>
<thead>
<tr>
<th>Sr.No.</th>
<th>Fractions</th>
<th>Mobile Phase</th>
<th>Volume in ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>n-Hexane:Chloroform(8:2)</td>
<td>10 ml</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>n-Hexane:Chloroform(6:4)</td>
<td>10 ml</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>n-Hexane:Chloroform(3:7)</td>
<td>10 ml</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>n-Hexane:Chloroform(1:9)</td>
<td>10 ml</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>Chloroform:Ethyl Acetate(7:3)</td>
<td>10 ml</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>Chloroform:Ethyl Acetate(5:5)</td>
<td>10 ml</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>Chloroform:Ethyl Acetate(3:7)</td>
<td>10 ml</td>
</tr>
<tr>
<td>8</td>
<td>8</td>
<td>Ethyl Acetate:Acetone(6:4)</td>
<td>10 ml</td>
</tr>
<tr>
<td>9</td>
<td>9</td>
<td>Ethyl Acetate:Acetone(5:5)</td>
<td>10 ml</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>Ethyl Acetate:Acetone(4:6)</td>
<td>10 ml</td>
</tr>
<tr>
<td>11</td>
<td>11</td>
<td>Acetone:Methanol(9:1)</td>
<td>10 ml</td>
</tr>
<tr>
<td>12</td>
<td>12</td>
<td>Acetone:Methanol(6:4)</td>
<td>10 ml</td>
</tr>
<tr>
<td>13</td>
<td>13</td>
<td>Acetone:Methanol(5:5)</td>
<td>10 ml</td>
</tr>
<tr>
<td>14</td>
<td>14</td>
<td>Acetone:Methanol(4:6)</td>
<td>10 ml</td>
</tr>
<tr>
<td>15</td>
<td>15</td>
<td>Methanol:Water(8:2)</td>
<td>10 ml</td>
</tr>
<tr>
<td>16</td>
<td>16</td>
<td>Methanol:Water(6:4)</td>
<td>10 ml</td>
</tr>
<tr>
<td>17</td>
<td>17</td>
<td>Chloroform:Methanol(9:1)</td>
<td>10 ml</td>
</tr>
<tr>
<td>18</td>
<td>18</td>
<td>Chloroform:Methanol(6:4)</td>
<td>10 ml</td>
</tr>
<tr>
<td>19</td>
<td>19</td>
<td>Chloroform:Methanol(5:5)</td>
<td>10 ml</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td>Chloroform:Methanol(2:8)</td>
<td>10 ml</td>
</tr>
<tr>
<td>21</td>
<td>21</td>
<td>Ethanol</td>
<td>10 ml</td>
</tr>
<tr>
<td>22</td>
<td>22</td>
<td>Methanol</td>
<td>10 ml</td>
</tr>
<tr>
<td>23</td>
<td>23</td>
<td>Water</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

Extracts prepared from the leaves of *A. mexicana*, along with streptomycin, were inoculated into wells punched in pre-seeded agar plates, as described for the agar well diffusion assay (Boyanova *et al.*, 2005; Singh *et al.*, 2009). After incubation at 37°C for 24 h, the zone of inhibition around the well was measured and recorded as the measure of antibacterial activity. Results depicted in Fig. 1 show that the *A. mexicana* leaf extract, methanolic (black bar) and cold aqueous (grey bar), exhibit significant antibacterial activity. The inhibitory zones obtained for methanolic and aqueous leaf extracts were found to be similar to streptomycin (empty bars), a comparison standard used in this study. These observations suggest that the *A. mexicana* leaf contains an antibacterial component of potentiality comparable to that of streptomycin.

**Determination of MIC**

The *A. mexicana* extracts, at concentrations of 200 µg/well and up to 1000 µg/well, were used for the antimicrobial potential determination. The MIC concentration was determined with the agar well diffusion assay by measuring the zone of inhibition. The MIC value for *A. mexicana* extracts was also determined by the broth dilution method (Washington & Sutter 1980). The inhibitory zones for agar well diffusion assay were measured (see Table 1). In the case of the broth dilution assay, bacterial growth was measured after 24 h incubation. Bacterial growth was found to decline with increasing concentrations of the extract. As concentrations of 800 mg/ml and above exhibited no bacterial growth, the said concentration was regarded as the MIC.

**Phytochemical analysis of the leaf extract**

The existence of phytochemicals in the methanolic extract of *A. mexicana* leaves was confirmed by various biochemical tests. Results shown in Table 2 suggest that the extract contained alkaloids, tannins, glycosides, carbohydrates, coumarins and flavonoids, but was found to be devoid of proteins, split amino acids and phenolics. The lack of amino acids, proteins and phenolics in the extract might have been due to the use of methanol as the solvent. The antibacterial potency observed for the extract above is likely to be associated with the phytochemical(s) detected in the extract.

**Purification and characterization of the active compound with antibacterial potency**

Thin Layer Chromatography (TLC)

After identifying phytochemicals that existed in the *A. mexicana* leaf methanolic extract, we then sought to purify the antibacterial compound with column chromatography. About 20 fractions were collected (see Materials & Methods) from the column and processed for TLC analysis, along with berberine chloride as the standard alkaloid. Table 3 shows various combinations of mobile phases applied for collecting fractions. Berberine is a quaternary ammonium salt from the protoberberine group of isoquinolinealkaloids. Previous studies had reported the use of berberine in China as a broad-spectrum anti-microbial medicine (Rubio-Pina & Vazquez-Flota, 2013). The *R* values for 20 fractions were recorded and compared with the *R* value obtained for berberine chloride (see Table 4). Fraction 12 of the extract showed identical *R* values with that of berberine chloride, enhancing likelihood of the existence of berberine in the extract. Hence, column fraction 12 of the leaves was processed for HPLC analysis.

High Performance Liquid Chromatography analysis of fraction 12
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matogram of the fraction 12 denotes three measurable
peaks with retention time 4.14, 4.5 and 5.39 shown in
Fig. 2. As expected, there was a major peak for pure
berberine chloride with retention time 2.99 shown in
Fig. 3. One minute difference was observed in the re-
tention time for peak 2 of the extract and pure berberine
chloride (Fig. 2 and Fig. 3). The second peak with reten-
tion time of 4.18 indicated the highest amount, hence it
was purified as peak 2 and used for elucidation of the
chemical formula.

NMR analysis of the HPLC purified peak 2

After HPLC analysis, which suggested the existence
of berberine in the methanolic extracts of A. mexica-
na, we decided to work on the structural differences of
the compound found within the HPLC peak 2 and the
pure berberine chloride. The spectrum obtained with
1H NMR (400 MHz, DMSO-d6) is shown in Fig. 4 of
A. mexicana HPLC peak 2. Analysis of the selected chem-
ical shifts from A. mexicana leaves HPLC peak 2 include:
δ 9.92 (s, 1H), 8.96 (s, 1H), 8.18 (d, J=9.2 Hz, 1 H), 7.99
(d, J=9.2, Hz, 1 H), 7.76 (s, 1 H), 6.16 (s, 2 H), 4.96 (t,
J=6.3 Hz, 2H), 4.08 (d, J=13.2 Hz, 6H), 3.21 (t, J=6.4
Hz, 2H) for molecular formula (C20H18NO4). It is evident
from 1H NMR spectrum shown in Figure 4 that two
singlets at δ value 9.92 and 8.96 of Hα are closer to N2,
whereas Hβ is away from N2 in the same ring. The Hc
and Hd aromatic protons are ortho coupled doublets at δ
value 8.18 and 7.99 (J=9.2 Hz), another ring inferred the
presence of isoquinoline moiety. The presence of anoth-
er aromatic ring having four substituents is indicated by
two singlets at δ value 7.76 and 7.06 of Hc and Hj,
respectively. Highly deshielded singlet at δ value 6.16 was
assigned to a methylene group CH2 (Cg), which is situ-
ated between two oxygen atoms linked to the aromatic
ring. In the spectrogram, two coupled triplets of Hj and
Hk of dihydro-pyridine rings can be seen. The Hj ap-
peared at δ value 3.21 and the spin – spin coupling con-
stant Jh–j in the range of 6.3–6.4 Hz. The singlet that
appeared at δ value 4.08 of 6 hydrogen is due to two
methoxy groups. All chemical shift values of respective
protons in the spectrum exactly matched with respective
groups. The structure elucidated from 1HNMR spec-
trum and molecular mass from mass spectrum analysis
through Mestrenova (C20H18CINO4) was exactly matched
with the spectrum of berberine and structure of berber-
ine with molecular formula which was obtained from the
PubChem compound. The structure elucidated from the
spectrogram, mestrenova and the PubChem was identi-
cal to berberine, shown in Fig. 5. This experiment con-
firmed the existence of berberine in the leaf methanolic
extracts of A. mexicana.

Functional demonstration of antibacterial potency for
purified berberine

Chang and coworkers (2003) carried out research on
A. mexicana and suggested that berberine, ephendrine,
hydrastine, canadine and palmatine are alkaloids present
in this plant and they may be responsible for its anti-
microbial activity. Hence, functional demonstration of anti-
bacterial potency of berberine (C20H18CINO4) from puri-
fied HPLC fraction was carried out. The HPLC purified
peak 2 from the extract that originated from fraction 12,
along with berberine purchased from Sigma Life Scienc-
es and streptomycin were used to testify the ability to inhibit the growth of clinical isolates. Antibacterial potency was tested with the agar well diffusion assay described above. Results shown in Fig. 1B demonstrated that both, the HPLC peak 2 and berberine, exhibited identical activity. The black bar denotes activity of berberine, while the light gray bar denotes identical activity of the HPLC purified peak 2, and the empty bar shows similar antibacterial activity of streptomycin. It is evident from the results shown in Fig. 1A and Fig. 1B that the antibacterial potency seen with the leaf extracts of A. mexicana and HPLC purified fraction were equal. The NMR studies demonstrated that the chemical present in the HPLC purified fraction is berberine and it would be suffice to say that the antibacterial potential demonstrated in the extract was likely due to berberine.

DISCUSSION

Previous studies (Chopra et al., 1986; Siddiqui et al., 2002; Osho & Adentunji, 2010; Rubio-Piña & Vázquez-Flota, 2013) had demonstrated that oil extracts of A. mexicana, at various levels of concentration, exhibited inhibitory effects towards filamentous fungi and non-filamentous fungus C. albicans, along with a few bacteria, such as Bacillus subtilis, Klebsiella pneumoniae, Staphylococcus aureus and Pseudomonas aeruginosa. With the use of agar well diffusion method evaluation of the antibacterial activity of crude extracts of aerial plant, root, stem, leaves and seed of A. mexicana against Bacillus subtilis, B. cerns, S. aureus, Streptococcus agalactiae, Enterobacter faecalis, Clostridium botulinum, Clostridium perfringens – i.e. the Gram positive bacteria, and E. coli, E. coli 157, Salmonella typhimurium, Proteus mirabilis, Proteus vulgaris, Klebsiella pneumonia, Pseudomonas aeruginosa – the Gram negative bacteria, was detected (Bhattacharjee et al., 2006; Rahman, 2009; Singh et al., 2009, Singh et al., 2009a; Osho & Adentunji, 2010; Syam Prasad & Dhanpal, 2010; Bhattacharjee et al., 2010; Alagesaboopathi & Kalaiselvi, 2012; Doss et al., 2012; Saranya, 2012). The present study aimed to spe-
cifically demonstrate antibacterial activity and further characterize the active ingredient from the *A. mexicana* leaves. This is a first report documenting that *A. mexicana* leaf methanolic extract contains berberine. In addition to earlier reports, the study presented here also reiterates the antibacterial potentiality of *A. mexicana* leaf extract, either aqueous or methanolic, against clinical isolates of *Bacillus cereus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* (Fig. 1A). Furthermore, we have successfully purified the active ingredient, elucidated its structure (Fig. 5) and confirmed its potency in comparison with that of the extract, as well as a standard broad spectrum antibiotic (Fig. 1B).

The *A. mexicana* derived compounds could play an important role in the development of drugs to control several diseases caused by various bacterial strains, particularly pathogenic *Pseudomonas aeruginosa* (Sharma & Nathawat, 1987; Sahu et al., 2012), and have an antiviral potential against HIV (Chang et al., 2003). In their studies, Chang and coworkers (2003) had shown that Protopines argemexicanas A and B obtained from *A. mexicana* exhibited a strong anti-HIV activity. The anti-HIV properties were ascribed to an alkaloid, 6-acetylhydrochelerythrine. The first medicinal application for *A. mexicana* has been reported in “the divine of farmers herb – root class”, Yinxi (2008). Earlier studies also indicated that the *A. mexicana* exhibit antifungal activity against filamentous and non-filamentous fungi (Siddiqui et al., 2002; Singh et al., 2009a; Osho & Adentunji, 2010; Syam Prasad & Dhanpat, 2010; More & Kharat, 2016). Our studies identified that the active ingredient present in the methanolic extracts of *A. mexicana* leaves was able to inhibit growth of Gram positive and Gram negative bacterial bacteria. The MIC obtained for both of these groups of bacteria was 800 mg/mL. Previous reports have indicated that when various solvents, such as ethanol, methanol, acetone, hexane, chloroform and water are used, the MIC for the *A. mexicana* extracts remained within a broad range of 125 mg/mL to 5.0 mg/mL (Rahman, 2009; Singh et al., 2009; Osho & Adentunji, 2010; Bhattacharjee et al., 2010; Alagesabooapathi & Kalaiselvi, 2012; Doss et al., 2012). A plausible explanation for the vast variation seen in the MIC from 125 mg/mL up to 5.0 mg/mL could be attributed to diverse solvents, choice of bacterium and its genetic make-up, along with the extraction generation procedure. Earlier studies have indicated that *A. mexicana* is likely to contain benzylisoquinoline alkaloids, such asbenzophenanthridines, protoberberines (berberine), protopines, Promexicine, methylhydrocorydalmine, jatrorrhizine, cumbammine, dl-tetrahydrocortisine and dihydrocortisine (Sharma & Nathawat, 1987; Singh et al., 2012; Rubio-Pina & Vazquez-Flota, 2013; Gobato et al., 2015). Sequential purification and characterization of the *A. mexicana* leaf extract was carried out using column chromatography, thin layer chromatography, high performance liquid chromatography and 1H NMR. When the column fractions were compared for their Rf values with berberine, it was found that fraction 12 did have an identical Rf with that of commercial berberine. The fraction was found to contain three peaks, out of which the second peak with a retention time of 4.1 was found to carry high concentration of the compound when compared to the other two peaks. Interestingly, the retention time for commercial berberine was 2.99. The delayed retention time for the major compound found in peak 2 might have been due to impurities that existed in fraction 12 when compared with commercial berberine purity. The HPLC purified peak 2 was then processed with 1H NMR. Interestingly, despite the differences obtained in their retention time, when analyzed with the Mestrenova software the peak 2 spectrum exhibited 100% similarity and was found to have an identical chemical formula, i.e. C_{20}H_{22}NO_{3} (Fig. 4 and Fig. 5). In previous studies by Bhattacharjee and coworkers (2010), the authors had purified the active antibacterial agent in the most potent fraction with methanol, and found it was an alkaloid, N-demethyloxysanguinarine. The berberine that exhibited broad spectrum antibacterial potency has been known to cause reversal of epithelial-to-mesenchymal transition (Chu et al., 2014). As the metastasis, a common cause of death due to cancer, largely relies on epithelial-to-mesenchymal transition, inhibiting this transition results in both, reduced metastasis and angiogenesis (Chu et al., 2014). The methanolic and cold aqueous extracts made from *A. mexicana* leaves and stem were shown to exert cytocidal effect on A549, SiH4 and KB immortalized cell lines (More & Kharat, 2016).

Functional characterization of antibacterial potency indicated that the compound purified from the *A. mexicana* leaf methanolic extract was berberine and exhibited comparable broad spectrum antibacterial activity, as depicted in Fig. 1B.

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**Conflict of interest**

Authors do not have any conflict of interest to declare.

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