EVALUATION OF ANTIBACTERIAL AND CARBONIC ANHYDRASE INHIBITORY POTENTIAL OF METHANOLIC EXTRACT OF NARDOSTACHYS JATAMANSI (D. DON) DC RHIZOMES

TAYYEB A. REHMAN1, SAEED AHMAD2, WAHEED MUMTAZ ABBASI1, ASHFAQ AHMAD3, MUHAMMAD BILAL1, MUHAMMAD MOHSIN ZAMAN1, AYMEN OWAIS GHAVRI1, ADEEL ARSHAD2 and KHALID AKHTAR1

1University College of Conventional Medicine, 2Department of Pharmacy, Faculty of Pharmacy and Alternative Medicine, The Islamia University of Bahawalpur, Bahawalpur, Pakistan
3Middle East Technical University, Ankara, Turkey

Abstract: Many antimicrobial drugs are going to become resistant to different pathogens, so the discovery of new antimicrobial products is an important public health concern. Nardostachys jatamansi (D. Don) DC (Valerianaceae) is an important traditional herbal medicine used as tranquilizer, CNS sedative, antiepilepsy, cardiotonic, diuretic, heptatonic, analgesic and in boils, itch and eye diseases. Carbonic anhydrase inhibitors are the potential source of treatment in case of glaucoma, hypertension and epilepsy and also used as diuretics. The aim of present study was to evaluate the antibacterial activity of methanolic extract of N. jatamansi against different bacterial strains and to estimate its carbonic anhydrase inhibitory potential. Antibacterial activity was evaluated by agar well diffusion assay and broth microdilution assay. The study revealed that N. jatamansi extract is sensitive to all tested bacterial strains. The zones of inhibition and MIC ranged from 8-22 at a concentration of 1-5 mg/mL and 0.3-0.6 mg/mL, respectively. Methanolic extract of N. jatamansi showed marked inhibition of carbonic anhydrase (IC 50 712.41 ± 0.001 µg/mL) when compared with standard acetazolamide. The results of the study suggest that N. jatamansi may be a valuable plant source of medicinally useful active compounds that can be helpful in bacterial infections and showed some relation with the traditional use of this plant in various diseases.

Keywords: Nardostachys jatamansi, antibacterial, carbonic anhydrase inhibitor

Nardostachys jatamansi (D. Don) DC; belongs to Valerianaceae family. It has an important place in traditional medicine in the Indian subcontinent and the Middle East, being used mainly as a tranquilizer and CNS sedative. Moreover, it is also used for gastrointestinal hyperactivity (1). The roots of Nardostachys jatamansi are used traditionally in the treatment of convulsive ailments, epilepsy, hysteria, heart palpitations, boils, diseases of the eyes, itch, etc. (2). In the Unani system of medicine, Sunbul-ul-tib (Nardostachys jatamansi) has been mentioned as a heptatonic, cardiotonic, diuretic and analgesic (3).

For the medicinal purpose, rhizomes of N. jatamansi are mostly used. Macroscopically, the rhizome of the plant is cylindrical and elongated in shape, and fine fibers in a network cover it. Phytochemical analysis showed the presence of alkaloids, amino acids, sugars and tannins in hot and cold methanolic extracts (4). Valeranone sesquiterpenes are the principal active constituents in N. jatamansi oil and it causes sleep induction (1). It contains many other sesquiterpenes including jatamansine, jatamansinol, oroseolol, oroselone, seselin, jatamol A and B, valeranal, nardostachyin, nardosinone, spirojatamol, jatamansic acid, nardostachone, calarenol, coumarin, jatamansin, xanthogalin, seychelane and seychellene (5).

Six Gram negative (Shigella dysenteriae, Corynebacterium striatum, Proteus vulgaris Escherichia coli, Pseudomonas aeruginosa, Kleb-
siella pneumoniae) and two Gram positive bacteria (Bacillus subtilis, Staphylococcus aureus) were included in the study. These bacterial strains were selected as these are the commonly infection causing bacteria in humans as Bacillus subtilis involve in the various allergic conditions of respiratory track, food poisoning and eye infections (6). Staphylococcus aureus is responsible for a variety of diseases including skin (boils, itch), soft tissue, bone, joint, food poisoning, cardiovascular, respiratory and wound infections. Pseudomonas aeruginosa can cause infections such as urinary tract infections, pneumonia, ear and eye infections and traumatic wound infections. Klebsiella pneumoniae causes urinary tract infections, wound infections, cholecystitis, meningitis and endocarditis. Escherichia coli is an opportunistic organism causing pneumonia and sepsis in immunocompromised host and meningitis (7).

Carbonic anhydrase inhibitors can be used for the treatment of many diseases. Carbonic anhydrase inhibitors are the first line treatments for glaucoma, cancer, osteoporosis, obesity, neurological disorders, as well as for gastric and duodenal ulcers. It may also act as diuretics and antiepileptic (8).

Many studies have been conducted to find the effects of N. jatamansi in CNS diseases (9). A literature search found that carbonic anhydrase inhibition and antibacterial activity of Nardostachys jatamansi methanolic extract against selected bacterial strains have not been reported so far.

In this study, antibacterial activity against some selected bacterial strains and carbonic anhydrase inhibition of N. jatamansi methanolic extract are described for the first time providing the basis for research to find new entities against different diseases.

EXPERIMENTAL

Reagents
Tris (Invitrogen: cat# 15504-020), HEPES (bioworld: cat#40820000-1), carbonic anhydrase (Sigma-Aldrich, C2624, P-Code: 1001584424), 4-nitrophenyl acetate (Sigma-Aldrich, N8130, lot#BCBK4587V), acetazolamide (Sigma-Aldrich, Lot BCBK5191V, P-Code 101400375, ≥ 99% powder), dimethyl sulfoxide (Merck, Germany), nutrient agar (Merck, Germany), nutrient broth (Merck, Germany), Ciprofloxacin (Noviday), Registration # 012066, Sami Pharmaceuticals (Pvt.) Ltd.

Apparatus
Micro plate reader (Synergy HT BioTek® USA), Digital rotary evaporator apparatus, (Heidolph Laboratory, Germany), pH meter (WTW series Inolab), digital weighing balance (Uni Bloc, Shimadzu, AUW220D).

Collection and identification of plant sample
Dried plant material was purchased from the local market and identified by the botanist, Dr. Sarwar, Lecturer, The Islamia University, Bahawalpur (Voucher No. 2205/L.S). The voucher specimen was deposited in Botany Department, The Islamia University, Bahawalpur, Pakistan.

Preparation of extract
The plant extract was prepared by maceration method. Plant material was powdered in the electric grinder; 100 g of dried powdered plant material was taken in the amber colored glass bottle and 400 mL of methanol was added to it. The material was soaked for 15 days with occasional shaking. After 15 days, the soaked material was filtered through muslin cloth and then by Whatman # 1 filter paper by using Buchner funnel. The process was repeated three times with 200 mL methanol to extract maximum contents and material (10). The solvent was evaporated by using rotary evaporator. The remainder was collected in little glass bottles and stored at 4°C until next use. It was the crude methnolic extract of N. jatamansi rhizomes.

A stock solution of freeze dried extract 5 mg plant extract/mL of DMSO was prepared and serial dilutions in the range of 0.5-5 mg/mL prepared from the stock solution.

Preliminary phytochemical screening
N. jatamansi rhizome extract was subjected to preliminary qualitative phytochemical screening for alkaloids (Dragendorff’s and Mayer’s test), flavonoids (sodium hydroxide), tannins (ferric chloride test) and phenols (ferric chloride test) (11).

Bacterial strains and growth media
Staphylococcus aureus (S.A) ATCC-6538, Pseudomonas aeruginosa (P.A) ATCC-9027, purchased from Microbiologics Inc. Escherichia coli (E.C), Klebsiella pneumonia (K.P), Shigella dysenteriae (S.D), Corynebacterium striatum (C.S), Bacillus subtilus (B.S), Proteus vulgaris (P.V) purchased from first fungal culture Bank of Pakistan (FCBP), Institute Of Agricultural Sciences, University Of The Punjab, Lahore, Pakistan; accession numbers were 12, 14, 72, 147, 174, 368, respectively. All the bacterial strains were grown on nutrient agar at 37°C for 24 h.
Synthetic antibacterial and carbonic anhydrase inhibitor

Ciprofloxacin (Novidat 200/100 mg/mL) was used as standard antibacterial drug and acetazolamide was used as a standard carbonic anhydrase inhibitor.

Preparation of inoculum

Nutrient agar media were prepared by dissolving 28 g of nutrient agar powder in 1000 mL of distilled water, heated until bubbles appeared and then sterilized at 121°C and 15 psi in autoclave for 15 min. Bacterial inoculums were prepared from 24 h old pure culture on nutrient agar. Bacterial colonies were grown in nutrient broth for 24 h. In 1 liter of distilled water, 8 g of broth was dissolved and kept in an autoclave (for sterilization) for 20 min at 121°C at 15 psi. In Erlenmeyer flasks, 50 mL of broth and 50 µL of culture stock solution was added and mounted on the horizontal shaker. After 24 h, cell turbidity was checked spectrophotometrically in comparison to that of 0.5 McFarland standard. Then, the inoculum was used for the antibacterial activity.

In vitro susceptibility tests

Agar well diffusion method

The agar well diffusion method was performed for the determination of antibacterial activity of *N. jatamansi* methanolic extract. Prior to being tested for the antibacterial activity, crude plant extract was dissolved in DMSO and stock solution of 5 mg/mL was prepared. The experiment was performed by the method of (12) with slight modifications. 20 mL of Mueller Hinton agar was placed in Petri dishes and allowed to solidify. A suspension of the microorganism of 60 µL was evenly spread on the surface of Mueller Hinton agar with sterile cotton-tipped swab. Wells of 6 mm in diameter were made on solid agar surface with the help of cork borer in each Petri dish. 20 µL of extract solution was added to each well. Petri dishes were placed in the incubator at 37°C for 24 h. After 24 h, the zones of inhibitions were measured to estimate the antibacterial activity. The experiment was done in triplicate and results were taken as an average of the three tests. The test was also performed with standard ciprofloxacin and methanol.

Broth micro dilution method

Antibacterial analysis and determination of minimum inhibitory concentration (MIC) of the extract and ciprofloxacin against different bacterial strains were performed by broth micro dilution method. This assay was carried out by the method of (13) with slight modifications. The test was performed in sterile 96-well micro plates. Total mixture volume in a well was 200 µL, contained 20 µL of methanolic extract solution and 180 µL suspension of bacterial culture. At 540 nm absorbance was measured and this was taken as pre read. Then for 16-24 hours, the plates were incubated at 37°C. After read was measured at 540 nm and the difference between pre read and after read was taken as an index of bacterial growth. All readings were taken as triplicate. Results are mean of triplicate (n = 3, ± S.E.M). Standard drug was ciprofloxacin and instead of test sample methanol was added in the assay as the negative control.

The % inhibition was calculated by the following formula

\[
\text{Inhibition (\%)} = 100 \times \frac{(X - Y)}{X}
\]

where

\[X = \text{absorbance in negative control with bacterial culture}\]
\[Y = \text{absorbance in test sample with bacteria}\]

Serial dilutions of the test samples were made to calculate the minimum inhibitory concentration. EZ-Fit5 Perrella Scientific Inc. Amherst USA software was used for MIC calculation.

Carbonic anhydrase inhibition assay

Carbonic anhydrase inhibition assay was performed according to (8) with slight modification. In this test, the formation of 4-nitrophenol was measured, that is a yellow color compound. 4-Nitrophenol was formed by the hydrolysis of 4-nitrophenyl acetate. The experiment was performed in 20 mM buffer of 7.4 pH containing Tris and HEPES. For each sample reaction well included following items: 140 µL of buffer, 20 µL of the freshly prepared solution of enzyme (0.1 mg/mL of deionized water) of purified bovine erythrocyte CA-II and 20 µL of the test compound. The test compound was incubated for 15 min at 25°C and pre-read was taken at 400 nm by using Synergy HT BioTek® USA micro plate reader.

The reaction started by the addition of 4-nitrophenyl acetate. 4-Nitrophenol acetate was added in 20 µL at the concentration of 0.7 mM, diluted in ethanol and incubated at the same conditions for 30 min and after read was taken at 400 nm. The reaction performed in triplicate with different concentrations. Assay was also performed with methanol for possible inhibition of enzyme as the extraction of plant was done with methanol. Percent inhibition was measured by formula given below;

\[
\text{% inhibition} = 100 - \left( \frac{\text{absorbance of test compound}}{\text{absorbance of control}} \right) \times 100
\]
Statistical analysis
All the measures were done in triplicate and results were expressed as the mean ± S.E.M. One-way ANOVA followed by Tukey post hoc test was used for statistical analysis. A p-value ≤ 0.05 was considered significant.

RESULTS
Preliminary phytochemical analysis of extract showed the presence of alkaloids, flavonoids, tannins and phenols.

The growth inhibition value of methanolic extracts of N. jatamansi on different bacterial strains

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Zone of inhibition (mm) mean ± SEM*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 mg/mL</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>12.6 ± 0.5</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>11 ± 0.5</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>10.3 ± 0.5</td>
</tr>
<tr>
<td>Shigella dysenteriae</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>Corynebacterium striatum</td>
<td>11 ± 0.5</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>11 ± 0.2</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>8 ± 0.5</td>
</tr>
</tbody>
</table>

*SEM, standard error of mean: *Mean ± SEM in each column differs significantly (< 0.05). Diameter of inhibition zones (Mean ± SEM) for the control was 32 ± 0.5; *Mean ± SEM in each column differs significantly (< 0.05). Diameter of inhibition zones (Mean ± SEM) for the control was 35 ± 1; *Mean ± SEM in each column differs significantly (< 0.05). Diameter of inhibition zones (Mean ± SEM) for the control was 35 ± 1; *Mean ± SEM in each column differs significantly (< 0.05). Diameter of inhibition zones (Mean ± SEM) for the control was 30 ± 1; *Mean ± SEM in each column differs significantly (< 0.05). Diameter of inhibition zones (Mean ± SEM) for the control was 26 ± 1; *Mean ± SEM in each column differs significantly (< 0.05). Diameter of inhibition zones (Mean ± SEM) for the control was 30 ± 1; *Mean ± SEM in each column differs significantly (< 0.05). Diameter of inhibition zones (Mean ± SEM) for the control was 30 ± 1.

Table 2. MIC of methanolic extract of N. jatamansi and ciprofloxacin against bacterial strains.

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>N. jatamansi MIC (mg/mL)</th>
<th>Ciprofloxacin MIC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus</td>
<td>0.4</td>
<td>1</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>0.3</td>
<td>1</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>0.5</td>
<td>0.015</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>0.4</td>
<td>1</td>
</tr>
<tr>
<td>Shigella dysenteriae</td>
<td>0.7</td>
<td>2</td>
</tr>
<tr>
<td>Corynebacterium striatum</td>
<td>0.6</td>
<td>8</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
<td>0.5</td>
<td>5</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>0.6</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 3. In vitro carbonic anhydrase inhibitory activity of N. jatamansi methanolic extract.

<table>
<thead>
<tr>
<th>Test substance</th>
<th>IC_{50} (Mean ± S.E.M)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. jatamansi</td>
<td>712.41 ± 0.001 µg/mL</td>
</tr>
<tr>
<td>Acetazolamide (Standard)</td>
<td>0.03 ± 0.00 µg/mL or 0.14 pM</td>
</tr>
</tbody>
</table>

*IC_{50} readings are mean ± S.E.M of 3 readings
is shown in Table 1. All bacterial strains showed marked antibacterial activity. The diameters of growth of inhibition zone of extract at various concentrations were between 8-22 mm. The diameters of the zone of inhibitions were decreased by reducing the concentration of extract. Ciprofloxacin showed zone of inhibitions in a range of 26-35 mm. The methanolic extract showed the highest inhibition against Bacillus subtilis and least against Shigella dysenteriae. However, methanol showed no inhibition against any bacterial strain. There was a significant difference between the zone of inhibition values of ciprofloxacin and extract (p < 0.05).

Table 2 summarizes the results of antibacterial activity as MIC through broth microdilution method. The MIC values of extract ranged from 0.3-0.7 mg/mL. Results showed that the highest inhibitory activity of the plant extract was found against Bacillus subtilis (MIC 0.3 mg/mL) and the lowest against Shigella dysenteriae (MIC 0.7 mg/mL). The results obtained from ciprofloxacin showed resistance to all selected bacterial strains representing MIC ranged from 0.015-8 µg/mL. There was a significant difference between MIC values of ciprofloxacin and extract (p < 0.05). It is evident from the results of the study that the highest the zone of inhibition, the lowest the minimum inhibitory concentration. In present study, the N. jatamansi extract showed highest zone of inhibition (22 mm) with the lowest MIC (0.3 mg/mL) against Bacillus subtilis. The lowest zone of inhibition was against Shigella dysenteriae (17 mm) with MIC (0.7 mg/mL).

In this study, N. jatamansi methanolic extract showed a marked inhibition of carbonic anhydrase (IC50 712.41 µg/mL) as represented in Table 3. Maximum inhibition of carbonic anhydrase (87%) was with the stock solution of the methanolic extract (1 mg/mL in DMSO). Methanol has no effect on carbonic anhydrase inhibition.

**DISCUSSION**

Drugs from natural sources are preferred because they perceived drug likeness and biological friendliness than synthetic compounds (14). Drug resistance due to an inappropriate and wide use of synthetic medicines is a major issue. Moreover, there are various side effects of these commercially available drugs (15). The study was conducted to investigate antibacterial activity of methanolic extract of N. jatamansi against S. aureus, P. aeruginosa, E. coli, K. pneumoniae, S. dysenteriae, C. striatum, B. subtilis, P. vulgaris, common clinical bacteria that can cause infection in humans. Antibacterial activity of different types of extract of N. jatamansi was reported against some bacterial strains (16), the screening of methanolic extract on all these bacterial strains and their minimum inhibitory concentrations (MICs) were not revealed before.

Based on agar well diffusion assay results of B. subtilis, S. aureus, K. pneumoniae showed the highest antibacterial activity while others showed moderate antibacterial activity. The similar findings were reported by (16) that the mixture of dichloromethane and methanol extract of N. jatamansi showed marked inhibition of B. subtilis, S. aureus, K. pneumoniae. However, Kumar (16) reported the lack of activity of essential oil and mixture of dichloromethane and methanol extract of N. jatamansi against P. aeruginosa and E. coli.

It has been frequently reported that antibacterial activity of medicinal plants is mostly due to the presence of alkaloids, flavonoids, tannins and triterpenoid in the plant extracts (17). The phytochemical evaluation of this extract has proved the existence of alkaloids, flavonoids and tannins in the methanolic extract, which might be the possible reason lying behind antimicrobial potential of this extract. The presence of alkaloids and tannins in methanolic extract of N. jatamansi was also reported in another work (4).

Antibacterial activity of extract against different bacterial strains was dose dependent. Activity was decreased by decreasing the concentration of extract. N. jatamansi extract has antibacterial effects may be by direct action of the extract on structure and metabolism of bacteria. The study was the first one that determined MIC of N. jatamansi extract. The role of MIC determination lies in the fact that lower the MIC, more chances of it being useful for medicinal purposes. The lower the doses required to achieve therapeutic effect have chances of lower toxicity and side effects (18).

*Staphylococcus aureus* skin and soft tissue infections cause minor boils or abscesses and can lead to severe infections as endocarditis (19). The roots of N. jatamansi are used traditionally in the treatment of boils and itch, etc. (2). Results of this study showed that N. jatamansi extract has marked inhibition against S. aureus (MIC 0.4 mg/mL) thus providing the basis for its traditional use in boils through antibacterial effects.

The results of present study presented the marked inhibition of carbonic anhydrase, so it can be speculated that traditional effects of N. jatamansi as diuretic, antiepileptic effect and in eye diseases may be due to carbonic anhydrase inhibition. N.
**jatamansi** extract can be used for clinical treatment of various diseases as a syrup. Formulation containing *N. jatamansi* as an important ingredient has reduced febrile convulsions in children during an experimental clinical trial (20). So, traditional antiepileptic effect of *N. jatamansi* that is proved in current study through *in vitro* carbonic anhydrase potential was also confirmed by a clinical trial. Moreover, anticancer effects of *N. jatamansi* that can be speculated from the current study due to carbonic anhydrase inhibitory potential is supported by cytotoxic activity of *N. jatamansi* extract against lung and prostate cancer cell lines (21).

**CONCLUSION**

*N. jatamansi* inhibited carbonic anhydrase sufficiently and showed significant antibacterial activity against different bacterial strains. Thus, the traditional use of it in various diseases is related to its reported activities. Moreover, the results of the study suggest that *N. jatamansi* may be a valuable plant source of medicinally active constituents that can be useful for the treatment of diseases.

**REFERENCES**


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