Isolation and Structural Determination of an Anti Bacterial Constituent from the Leaves of Cassia alata Linn.

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By different solvent extractions and chromatographic techniques an antibacterial constituent was isolated from leaves of Cassia alata Linn. Infra red spectroscopy, mass spectroscopy and nuclear magnetic resonance studies showed that the isolated compound was chemically 3,4 dihydroxy cinnamic acid. In vitro antibacterial activity of 3,4 dihydroxy cinnamic acid was studied against four Gram-positive and four Gram-negative bacteria using disc diffusion method. Minimum inhibitory concentration (MIC) of 3,4 dihydroxy cinnamic acid was also recorded against those bacteria by serial dilution technique. Kanamycin was used as positive control. Results showed that 3,4 dihydroxy cinnamic acid had antibacterial activity against the tested bacteria.

Keyword: Cassia alata Linn., Gram-positive and Gram-negative bacteria, 3,4 dihydroxy cinnamic acid, kanamycin

1. Introduction
Several plants have shown anti–microbial activity. Few of them are Azadirachta indica¹, Clausena anisata², Amona glabra³, Semecarpus anacardium⁴, Garcinia mangostana⁵, Aegle marmelos⁶, Santolina chamaecyparissus⁷, Terminalia belerica⁸, etc.
Cassia alata Linn. (family, Caesalpiniaceae) is an erect tropical annual herb with leather compounded leaves. It grows everywhere in the state of West Bengal, India up to 6 ft tall. It has different names like ringworm weed in English, dadmari in Hindi and cakramard in Sanskrit. Its therapeutic values as mentioned in Ayurvedic text⁹ are: Leaves are antiparasitic, used in eczema, bronchitis, asthma, ringworm and in poisonous insect bites. Bark is used to treat skin diseases. Extract of aerial parts is CNS depressant, diuretic and anti-inflammatory.
In 1998 Sakharkar and Patil found antimicrobial activity of Cassia alata Linn¹⁰. We also noted that leaves of Cassia alata Linn. could inhibit growth of Staphylococcus aureus¹¹. We thus undertook experiments to isolate the antibacterial constituent from Cassia alata Linn and to
elucidate its structure. Further, antibacterial activity of the isolated compound was also studied against few Gram-positive and Gram-negative bacteria. In present communication we report the results of those experiments.

2. Materials and Methods

2.1 Plant material
Leaves of *Cassia alata* Linn. were collected from the medicinal plants garden of the University of North Bengal, Siliguri, India and authenticated by Prof. A. P. Das of the department of Botany 1of the said University. A voucher specimen was kept in the department for future reference.

2.2 Extraction and Isolation

1. **First step:** Leaves of *Cassia alata* Linn. were sundried and powdered. 50g of this powder were extracted with 500 ml of 10 : 1 (v/v) acetone – ethyl alcohol mixture for 1h on a rotary shaker. It was then centrifuged. Supernatant was collected and evaporated to dryness. Dry brown mass was obtained.

2. **Second step:** Dry brown mass was refluxed with 100 ml of 1(N) HCL for 1h on a water bath at 100 degree centigrade. It was cooled and centrifuged. Supernatant was evaporated to dryness.

3. **Third step:** Dry brown mass thus obtained from the supernatant was extracted with 50 ml of a mixture of water and isobutanol (2 : 1 v/v) on a rotary shaker for 1h. Isobutanol layer was separated from water layer. It was evaporated to dryness.

4. **Fourth step:** Brown mass obtained was dissolved in 10 ml methanol and subjected to column chromatography using silica gel Gas adsorbent. 5 bands were separated. Bands were collected in separate beakers. Elution was done by 50% methanol – chloroform mixture. Third band had antibacterial activity against *Staphylococcus aureus*.

5. **Fifth step:** Eluant of third band was evaporated to dryness. The dry mass was extracted with 15 ml ethyl acetate for 10 minutes. It was then filtered. With filtrate polyamide column chromatography was done. Elution was made by ethyl formate: formic acid mixture (100: 5 v/v). Three bands were separated. Second band showed antibacterial activity against *Staphylococcus aureus*.

6. **Sixth step:** Eluant of second band was evaporated to dryness. Repeated crystallization was done from ethyl acetate–cyclohexane (50:50, v/v) mixture. Crystals obtained. Yield was 3.6 mg.

2.3 Homogeneity of the active compound
This was ascertained by silica gel- G thin layer chromatography by using the following solvent systems: Acetone : methanol - 50 : 50; n-butanol : acetic acid : water - 80 : 10 : 10; Chloroform : methanol : water - 60 : 20 : 20

2.4 Structure determination
FT-IR spectrum of the sample was taken in KBr pellets using Shimadzu FT-IR 8300 Spectrophotometer. NMR spectrum was taken using Bruker AVH 300 Spectrometer operating at 300 MHz (for $^1$H) and 75 MHz (for $^{13}$C) and in solvent, as indicated. $^{13}$C NMR spectrum was run in $^1$H-decoupled mode. The High Resolution Mass Spectral data for the compound was obtained in Mass Spectrometer (Model: Micromass Q-Tof Micro), run under Electron Spray Ionization (ESI) Positive Mode. Melting point was observed in an open sulfuric acid bath and is uncorrected.

2.5 Anti-bacterial Screening

2.5.1 Bacteria
Four Gram-positive strains, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 19659, *Streptococcus pyogenes* MTCC 512 and *Bacillus megaterium* MTCC 302 as well as four Gram-negative strains, *Shigella flexneri* MTCC 678, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Salmonella typhi* MTCC 733 were used in this study. Bacterial strains were collected from the department of Microbiology, North Bengal Medical College Hospital, Siliguri, West Bengal, India and maintained on nutrient agar slants at 4°C.

2.5.2 Media
Nutrient agar media (Difco laboratories) pH 7.2 and nutrient broth media (Difco laboratories) pH
26.8 were used for antibacterial screening and MIC (minimum inhibitory concentration) determination respectively.

2.6 In Vitro Antimicrobial activity of the isolated compound

In vitro antibacterial screening was carried out by disc diffusion method\(^\text{12}\). According to this method, 20 ml quantities of nutrient agar were placed in a petri dish with 0.1 ml of \(10^{-2}\) dilution of bacterial culture of 20 hours old. Filter paper discs (6 mm diameter) impregnated with 30 µg per disc of the isolated compound was placed on bacterial seeded plates. The isolated compound was soluble in water. Blank disc impregnated with water was used as negative control. Zone of inhibition was recorded after 20 hours of incubation at 37\(^\circ\)C. Diameters of zone of inhibition produced by the isolated compound were compared with that of standard antibiotic kanamycin under same experimental conditions.

<table>
<thead>
<tr>
<th>Test organism</th>
<th>Zone of inhibition (diameter in mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3,4 dihydroxy cinnamic acid (30 µg/disc)</td>
<td>Kanamycin (30 µg/disc)</td>
</tr>
<tr>
<td>Gram positive</td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>25 ± 1.1</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>73 ± 1.2</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>22 ± 1.4</td>
</tr>
<tr>
<td>Bacillus megaterium</td>
<td>21 ± 1.0</td>
</tr>
<tr>
<td>Shigella flexneri</td>
<td>16 ± 1.0</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>12 ± 0.9</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>20 ± 1.1</td>
</tr>
<tr>
<td>Salmonella typhi</td>
<td>18 ± 1.0</td>
</tr>
</tbody>
</table>

Data for control disc has not been given here as it had no zone of inhibition. Results were in mean ± SEM (n = 5)

2.7 Minimum inhibitory concentration (MIC) determination

Minimum inhibitory concentration is defined as the lowest concentration of antibiotic completely inhibiting visible growth of bacteria after 18 – 24 hours of incubation at 37\(^\circ\)C. This was done by the method of Mosaddik and Haque\(^\text{13}\). According to this method, the isolated compound (1.0 mg) was dissolved in 2 ml nutrient broth media to obtain a stock solution of concentration 500 µg/ml. 3 drops of Tween 80 was added in nutrient broth to facilitate dissolution. Serial dilution technique was followed to obtain 250 µg/ml concentration of the isolated compound. One drop (0.02ml) of prepared suspensions of organism (10\(^6\)organism/ml) was added to each broth dilution. These dilutions were then incubated for 20 hours at 37\(^\circ\)C. Growth of bacteria was examined by noting turbidity of the solution. The nutrient broth media with 3 drops of Tween 80 was used as negative control while kanamycin under same experimental condition was used as positive control.
2.8 Statistical analysis
The values were expressed as mean ± SEM and was analyzed using one-way analysis of variance (ANOVA) using Statistical Package for Social Sciences (SPSS) 20th versions. Differences between means were tested employing Duncan’s multiple comparison tests and significance was set at p < 0.05.

3. Results and Discussion
3.1 Homogeneity of the active Compound
In all cases of thin layer chromatographic experiments using three different solvent systems single spot was obtained. Thus, it was a single compound.

Fig 2: IR spectrum of the isolated compound

3.2 Structure Elucidation
The compound was a pale yellow solid, mp. 218-221°C. NMR data were as follow : The 1H-NMR (D6-DMSO): δ 6.16 (d, 1H, J = 15.9 Hz), 6.75 (1H, d, J = 7.4 Hz), 6.95 (dd, 1H, J = 8.1 & 2.1 Hz), 7.02 (s, 1H), 7.41 (d, 1H, J = 15.9 Hz), 9.12(br. s, 1H), 9.52 (br. s, 1H), 12.11 (br. s, 1H) ppm. Its 13C-NMR (D6-DMSO): δ 115.1, 115.6, 116.2, 121.6, 126.2, 145.1, 146.0, 148.6, 168.4. From 1H-NMR spectral data (Figure-1), it appeared that there were three aromatic protons, two olefinic protons and three broad singlets. The coupling patterns of the aromatic protons primarily indicated one ortho coupled doublet and one ortho-meta coupled doublet of doublet (J = 8.1 & 2.1 Hz) and the other was possibly a meta-doublet, though appeared as a singlet (δ = 7.1 ppm). On the other hand, the olefinic protons with coupling constant, J = 15.9 Hz, indicated that the double bond was in trans configuration. Since there were three aromatic protons as seen by 1H 3NMR spectral data, the other three positions of the aromatic ring might be substituted. Only one aromatic ring was considered because of low molecular mass of the compound. Also, there were only nine chemically non-equivalent carbons according to 13C-NMR spectrum. As one substituent might be a C–C double bond, there were two other positions, might be substituted with two hydroxyl group (OH) that appeared as broad singlets. Out of three broad singlets, one broad singlets at δ=12.11 ppm could be assigned for the carboxylic (COOH) proton. The carboxylic acid group might be attached with the C–C double bond, leading to propose the assigned structure as dihydroxy cinnamic acid. The FT-IR (KBr) absorption maxima, shown in Figure-2, (V_max) at 3423, 3179, 1675, 1657, 1603 cm⁻¹ also suggested the presence of hydroxyl, conjugated carboxyl and double bonds. Considering that the compound could be a dihydroxy cinnamic acid and based on the coupling pattern of three aromatic protons (one ortho-doublets, one ortho-meta doublet of doublet and one meta-doublet), theoretically five possible structures, as shown in figure 3, might be proposed :

The 13C-NMR spectral data (Figure-4) showed that there are nine chemically non-equivalent carbons, out of which three carbons were assigned for the acrylic acid side chain carbons (–C=C–COOH). Therefore, all aromatic ring hydrogens were non-equivalent. 3,5-Dihydroxycinnamic acid is having the axis of symmetry and thus it might have only four chemically and magnetically non-equivalent carbons. Accordingly, this structure (3,5-dihydroxycinnamic acid) might be ruled out. Out of other four structures, the literature value of the melting point of 3,4-dihydroxycinnamic acid (mp 223-25°C) fairly matched with the observed melting point of the compound (218-221°C). Its coupling pattern was shown below with the possible coupling of the aromatic hydrogens as well as the trans-configuration of the carbon–carbon double bond, which was observed and
calculated to be $J=15.9$ Hz). The trans-configuration of the carbon–carbon double bond was assigned based on the fact that in the case of cis-configuration, the coupling constant ($J$) would have been within 6–12 Hz. The spin-spin couplings between hydrogens were clearly shown in figure - 5 for the aromatic hydrogens (marked as $H_a$, $H_b$ and $H_c$) as well as for the carbon–carbon double bonds, marked as $H^\alpha$ and $H^\beta$.

Structure of the compound was further corroborated by the High Resolution Mass Spectral (HRMS) data (Figure-6), run under Electron Spray Ionization (ESI) Positive Mode. In HRMS, the exact mass for compound with mf $\text{C}_9\text{H}_8\text{O}_4\text{Na} [\text{M}^+\text{Na}]$ was calculated to be 203.1472 and observed as 203.1622. Therefore, the structure of the isolated compound, shown in figure-7, may be assigned as 3,4-Dihydroxycinnamic acid.

Antibacterial activity of 3,4 dihydroxy cinnamic acid was studied. Results, given in Table- 1, showed that 3,4 dihydroxy cinnamic acid in disc concentration 30 µg produced more zone of inhibitions (in between $21 \pm 1.0$ and $25 \pm 1.1$) for Gram-positive bacteria than Gram-negative bacteria (zone of inhibitions, $12 \pm 0.9$ and $20 \pm 1.1$). Kanamycin, however, produced more zone of inhibitions for both Gram-positive (29 ± 1.2 to 32 ± 1.3) and Gram-negative (20 ± 1.0 to 29 ± 1.4) bacteria under same experimental conditions.

Table 2: Minimum inhibitory concentration (MIC) of 3,4 dihydroxy cinnamic acid and kanamycin

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>MIC values of 3,4 dihydroxy cinnamic acid (µg/ml)</th>
<th>MIC values of kanamycin (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>Bacillus megaterium</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>Shigella flexneri</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>32</td>
<td>16</td>
</tr>
<tr>
<td>Salmonella typhi</td>
<td>10</td>
<td>8</td>
</tr>
</tbody>
</table>

Data for negative control containing solvent only has not been given here as it had no MIC value.

Resistance to antibiotics is becoming an increasingly difficult problem in the management of bacterial infections\[14\]. The situation is particularly critical for *Staphylococcus aureus* where methicillin – resistant (MRSA) and vancomycin intermediate resistant (VISA) strains have emerged, that are also frequently resistant to multiple classes of antibiotics\[15\]. Recent reports of high – level vancomycin resistance in MRSA as a result of acquisition of the vanA determinant from enterococci\[16\] and the emergence of MRSA resistant to linezolid\[17\] are further disturbing trends in the evolution of antimicrobial resistance in *Staphylococcus aureus*. Thus, Al – Bari *et al.*,\[18\] asserted that a large number of antibacterial agents have been discovered but pathogenic bacteria are constantly developing resistance to these agents. Due to this, Rahman *et al.*,\[19\] stated that the life threatening bacterial infection has been increased worldwide and is becoming an important cause of morbidity and mortality. Under the circumstances, stress is given to search for new antibacterial agent\[20\]. Research is going on in this direction and is extended even in the field of medicinal plants to develop safer antibacterial drugs\[21\].
We, in our laboratory, when screened medicinal plants for their antibacterial property noted that leaves of *Cassia alata* Linn. Could inhibit growth of *Staphylococcus aureus*. We intended to isolate the active compound from leaves of *Cassia alata* Linn. By different solvent extractions followed by chromatography an active compound was isolated. Elucidation of structure of the active compound was undertaken by spectroscopic and other analytical data. IR spectroscopy, mass spectroscopy and nuclear magnetic resonance data suggested that the active compound was chemically 3,4 dihydroxy cinnamic acid.

**Fig 3:** Possible structures of the isolated compound

**Fig 4:** 13C NMR spectrum of the isolated compound

*In vitro* antibacterial activity of 3,4 dihydroxy cinnamic acid was carried out by disc diffusion method against four Gram-positive and four Gram-negative bacteria. Zone of inhibitions suggested that 3,4 dihydroxy cinnamic acid had antibacterial activity against the tested bacteria though it was more effective for Gram-positive bacteria than Gram-negative bacteria. Control drug kanamycin, however, produced larger zone of inhibitions under same experimental conditions.

**Fig 5:** Spin-spin couplings between hydrogen in the structure of the isolated compound.

Minimum inhibitory concentrations (MIC) of 3,4 dihydroxy cinnamic acid against the tested bacteria were more in comparison to kanamycin. But in case of *Staphylococcus aureus* MIC value was recorded same (4 µg/ml) for both 3,4 dihydroxy cinnamic acid and kanamycin. Therefore, from the present study it may be concluded that 3,4 dihydroxy cinnamic acid isolated from the leaves of *Cassia alata* Linn. had anti-bacterial activity. 3,4 dihydroxy cinnamic acid is a phenolic compound. Our findings thus support the earlier observation that phenolic compounds exert antibacterial property[22]. Mechanism of antibacterial activity of 3, 4 dihydroxy cinnamic acid needs to be explored. Work in this direction is now in progress.
4. Conclusion

3,4 dihydroxy cinnamic acid was isolated from the leaves of *Cassia alata* Linn. and found having antibacterial activity against four Gram-positive bacteria viz. *Staphylococcus aureus*, *Bacillus subtilis*, *Straptococcus pyogenes* and *Bacillus megaterium* as well as four Gram-negative bacteria like, *Shigella flexneri*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella typhi*. Anti-bacterial activity was comparable to that of known antibiotic kanamycin.

5. References


