Comparative Analysis of *Cleistanthus collinus* Aqueous Leaf Extract and Fractions for its Antibacterial Potential

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**Background:** Drug resistance property of disease causing pathogens is a serious issue in the field of medicine and so further attempts to search for new antimicrobial agents from plant source is the need of the hour.

**Objectives:** The purpose of this study was to examine the preliminary phytochemicals and investigate the antibacterial potential of *C. collinus* (Euphorbiaceae) aqueous extract and fractions.

**Materials and Methods:** *C. collinus* plant leaf material was dried and the hot aqueous extract was prepared. All the fractions obtained from serial and direct extraction methods were subjected to the preliminary phytochemical screening and characterization. Antibacterial activity and minimum inhibitory concentration of *C. collinus* crude aqueous extract and its fractions were analyzed against selected pathogens.

**Results:** Preliminary phytochemical screening, UV-Vis spectrum analysis clearly differentiate crude leaf extract and fractions. Among the tested plant extract and fractions, ethyl acetate and hexane fractions showed profound antibacterial activity. Serial fraction of hexane showed higher antibacterial activity against *Listeria monocytogenes, Klebsiella pneumoniae, Escherichia coli* and *Vibrio cholerae* (21 - 40 mm zone of inhibition and MICs 36.5 - 70.5 μg). *V. cholerae* was highly susceptible to all the fractions and extract of *C. collinus*.

**Conclusions:** Aqueous extract and its serial fractions of *C. collinus* exhibited bactericidal activity against tested pathogens. This study confirms the presence of promising Phyto-constituents in *C. collinus* leaf material which could be exploited for the further pharmacological studies to develop a drug to control the infectious disease causing pathogens.

**Keywords:** Antibacterial; Cleistanthus; Leaf extract; MIC; Pathogens

1. **Background**

   World Health Organization (WHO) reported that the infectious diseases are responsible for over 50% deaths worldwide mainly in tropical and developing countries. Unavailability of current medicine is the most important reasons for the people in these areas to rely on traditional medicine (1, 2). A lot of recent studies demonstrated significant agreement between the traditional use of the plant parts by native people in the treatment of specific symptoms and experimental antimicrobial and anti-cancer activities in the laboratory (3). Botanicals have been used in the treatment of several diseases caused by bacterial pathogens. In the recent years, bactericidal agents derived from the plants have been getting attention in drug discovery, as most of antibiotics under usage have shown the ineffectiveness against several pathogens, due to increasing drug resistance. The bactericidal activity has been reported in various plant constituents such as phenols, quinines, flavones, flavonoids, flavonols, tannins, terpenoids, essential oils and alkaloids etc. (4).

*Cleistanthus collinus* (Euphorbiaceae) is a small tree species naturally distributed in the dry forests of the south and central parts of Asian countries (5, 6). *C. collinus* leaves contain saponin, tannin and oduvin moreover the poisonous effect attributed to oduvin usually occurs after drinking the decoction of the leaves of *C. collinus* leads to death within 1 - 3 days (7, 8). *C. collinus* is a rich source of lignans of which cleistanthin A and B was reported as having the property of cytotoxicity, anticancer and larvicidal activity (9, 10). It is also used as washing agent for clearing septic wounds, cure fungal and bacterial diseases (11). Perusal of literature revealed that there are merely two reports (12, 13) have been encountered in the assay of antibacterial activity of this plant. With this backdrop information we had carried out an assay to analyze preliminary phytochemical screening, UV-Vis absorption spectrum and antibacterial activity of other solvent extracts fractioned in serial and direct manner from *C. collinus* leaves.
2. Objectives
The purpose of this study was to analyze the antibacterial activity and preliminary phytochemicals of direct and serial fractions of C. collinus leaf extract against selected pathogens.

3. Materials and Methods

3.1. Plant Collection and Extraction
The C. collinus plant material was collected from plains of Virallimalai, Tamilnadu, India. The plant material was shade dried and powdered to precede extraction. Aqueous extraction of the sample was prepared by adding 500 g of leaf powder in 2000 mL distilled water (1:4 w/v ratios) and boiled well for 20 minutes. Next, the crude material was filtered through whatman no.1 filter paper, and the filtrate was concentrated in freeze dryer. 2.0 g of crude extract powder was reconstituted in 100 mL of distilled water and 100 mL of butanol for the serial fractionation. The mixer was taken in a separating funnel for the collection of fractions. Similarly, the serial fractionation process was continued by adding an equal volume of ethyl acetate, dichloromethane and hexane with the remains obtained in each step. In the same way the direct fractions were collected from an aqueous extract of C. collinus by liquid-liquid separation method using the same solvent system.

3.2. Phytochemical Screening and UV-Vis Absorption Spectrum Analysis
The preliminary phytochemical analysis was carried out for the aqueous extract and all the fractions collected by both serial as well as direct method using standard procedure by Odebiyi and Sofowora method (14). UV-Vis wave length scanning analysis was carried out for all the extracts and its fractions using UV-Vis spectrophotometer (UV2310 Techcomp).

3.3. Test Pathogens
Eight representative bacterial strains (Escherichia coli MTCC-433, Pseudomonas aeruginosa MTCC-741, Staphylo-
coccus aureus MTCC-1430, Shigella flexneri MTCC-1457, Salmonella typhi MTCC-733, Klebsiella pneumoniae MTCC-432, Vibrio cholerae MTCC-3940 and Listeria monocytogenes MTCC-1143) were used for antibacterial susceptibility test. They were procured from the Microbial Type Culture Collection (MTCC) center of IMTECH, Chandigarh, India.

3.4. Antibacterial Activity and Assessment of MIC
The Kirby-Bauer disc diffusion assay was followed to determine the antibacterial activity of the aqueous crude extract and its fractions of C. collinus plant with positive and negative controls as described by Bauer et al. (15). MICs were performed for the extract and fractions (which produce ≥ 8 mm diameter zone of inhibition in disc diffusion method) in 96-well micro-plates (16).

4. Results
The C. collinus aqueous leaf extract was prepared and 2 g of this was used up for serial and direct fractionations with solvents like n-butanol, ethyl acetate, dichloromethane and hexane. Serial extraction and fractionation yielded various quantities of residue. The residue obtained from n-butanol fraction was 171 mg, followed by an ethyl acetate fraction, dichloromethane fraction, hexane solvent yielded 84, 67, 22 mg of residue respectively. The intermediate layer obtained at every fractionation was discarded. Final remain was also concentrated to get 440 mg of residue. 210, 286, 152 mg of final residues of n-butanol, ethyl acetate, dichloromethane, and hexane were collected from C. collinus aqueous extract by direct fractionation.
All the fractions obtained through direct method as well as the fractions obtained through serial fractionation of aqueous extract were screened for the preliminary phytochemicals like tannins, terpenoids, flavonoids, saponins, glycosides, steroids, phlobatannins and alkaloids (Table 1).

The C. collinus aqueous extract and its fraction were subjected to UV-Vis spectroscopy analysis. UV-Vis absorption spectrum of aqueous extract contains three major peaks (334,370 and 385 nm). First immediate fraction of aqueous extract was prepared using n-butanol, which produced totally eleven peaks, of them five major (335, 355, 366, 372 and 383 nm) and six minor peaks were observed.

<table>
<thead>
<tr>
<th>Phytochemical</th>
<th>Aqueous n-Butanol a</th>
<th>Ethyl acetate a</th>
<th>Dichloromethane a</th>
<th>Hexane a</th>
<th>Final residue</th>
<th>n-Butanol b</th>
<th>Ethyl acetate b</th>
<th>Dichloromethane b</th>
<th>Hexane b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tannins</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Steroids</td>
<td>-</td>
<td>+</td>
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<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Flavonoids</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>Glicosides</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Phlobatannins</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>Alkaloids</td>
<td>-</td>
<td>+</td>
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<td>-</td>
<td>-</td>
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<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Saponins</td>
<td>+</td>
<td>-</td>
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<td>+</td>
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</tbody>
</table>

a Serial Fractionation from Crude Aqueous Extract of C. collinus.
b Direct Fractionation from Crude Aqueous Extract of C. collinus.
Similarly the subsequent fraction of ethyl acetate showed only five peaks, followed by four peaks was obtained in dichloromethane fraction. Hexane fraction of the extract contains few metabolites which were having its absorption maximum at UV-ranges (222, 234, 253, 264, 278, 305 and 376 nm). However, direct single solvent based fractionation of aqueous extract yielded a unique kind of UV-Vis absorption spectrum. It is obvious that ethyl acetate solvent based direct fractionation eluted more number of compounds from the aqueous extract and so produced more number of peaks which were absent while preparing the fraction by the serial fractionation method. Although, n-butanol and dichloromethane solvents based direct fraction did not show any remarkable changes in the absorption spectrum. It also indicates that the same kind of metabolites might have been eluted in both types of fractionation. In the case of hexane direct fraction, metabolites having its absorption maximum at visible range (533, 599, and 654 nm) were obtained which could not be eluted during the serial fractionation method (Figure 1 A and B).

The aqueous extract and its fractions of C. collinus plant were subjected to bioassay test against pathogenic bacteria. The antibacterial potential was measured as the diameter of the zone of inhibition; the data were furnished in Table 2 and Figure 2. The aqueous extract of C. collinus showed significant antibacterial activity against S. typhi, K. pneumoniae, E. coli and V. cholerae among the tested bacteria. Antibacterial activity was found to be more in the fractions containing secondary metabolites. Remarkable antibacterial activity was observed in C. collinus fractions obtained from hexane and ethyl acetate solvents than aqueous extract. The positive controls Co-trimoxazole and Tetracycline consistently displayed superior potency and being as a known marker to compare the activity of aqueous extract and other solvent fractions. Discs impregnated with only solvents (negative control) did not show any bioactivity.

![UV-Vis Absorption Spectrum of C. collinus Extracts and its Fraction](image)

**Figure 1.** UV-Vis Absorption Spectrum of C. collinus Extracts and its Fraction

**Table 2.** Antibacterial Activity of C. collinus Extract and its Fractions

<table>
<thead>
<tr>
<th>Fractions and extract, 1 mg/disk</th>
<th>Zone of Inhibition, mm</th>
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<tbody>
<tr>
<td></td>
<td>Lm</td>
</tr>
<tr>
<td>Aqueous Crude Extract</td>
<td>-</td>
</tr>
<tr>
<td>Butanol Fraction</td>
<td>S</td>
</tr>
<tr>
<td>D</td>
<td>-</td>
</tr>
<tr>
<td>Ethyl Acetate Fraction</td>
<td>S</td>
</tr>
<tr>
<td>D</td>
<td>-</td>
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<tr>
<td>Dichloromethane Fraction</td>
<td>S</td>
</tr>
<tr>
<td>D</td>
<td>-</td>
</tr>
<tr>
<td>Hexane Fraction</td>
<td>S</td>
</tr>
<tr>
<td>D</td>
<td>-</td>
</tr>
<tr>
<td>Co-trimoxazole, 25 μg b</td>
<td></td>
</tr>
<tr>
<td>Tetracycline 30 μg b</td>
<td>24</td>
</tr>
<tr>
<td>Butanol c</td>
<td>-</td>
</tr>
<tr>
<td>Ethyl Acetate c</td>
<td>-</td>
</tr>
<tr>
<td>Dichloromethane c</td>
<td>-</td>
</tr>
<tr>
<td>Hexane c</td>
<td>-</td>
</tr>
</tbody>
</table>

**Abbreviations:** S, Serial Fraction; D, Direct Fraction; Lm, Listeria monocytogenes; Sf, Shigella flexneri; St, Salmonella typhi; Kp, Klebsiella pneumoniae; Ec, Escherichia coli; Sa, Staphylococcus aureus; Pa, Pseudomonas aeruginosa; Vc, Vibrio cholerae.

b Positive Control.

c Negative Solvent Control.
C. collinus leaf extract and fractions which exhibit antibacterial potential with the zone of inhibition ≥ 8 mm diameter were subjected to determine MICs quantitatively (Table 3). Hexane fraction in the serial extraction showed off the highest inhibitory effect at lowest concentration (≥36.5 - ≤ 70.5 μg/mL) against *K. pneumoniae*, *E. coli*, *V. cholerae* and *L. monocytogenes* followed by other serial and direct fractions and extract. Both serial and direct fractions and extract displayed good inhibitory effect against *V. cholerae*. Among them the hexane, ethyl acetate serial fraction and aqueous extract inhibited the growth of the target pathogen *Vibrio* even at low (53.5, 115 and 177 μg/mL) concentrations.

5. Discussion

The antimicrobial property of specific plant part of particular plant species may be due to the presence of one or more bioactive compounds such as tannins, terpenoids, flavonoids, sapopins, glycosides, steroids, phlobatanins and alkaloids (17, 18). The extract and fractions of *C. collinus* have been proven for the occurrence of various phytochemical groups like tannins, terpenoids, flavonoids, sapopins, glycosides, steroids, phlobatanins and alkaloids,
which are secondary metabolites produced and stored in different tissues of the plant. The aqueous extract of *C. collinus* leaf contains five major metabolites. This is in accordance with the finding of Suman et al. (13). Aqueous extract was found to be moderately promising in controlling *S. typhi*, *K. pneumoniae*, *E. coli* and *V. cholerae* growth but failed to control the growth of *L. monocytogenes*, *S. aureus*, *P. aeruginosa* and *S. flexneri*. Aqueous extract highly inhibits the growth of *V. cholerae* at the lowest concentration. Previously, it was described that the *C. collinus* cold extracts from aqueous, acetone, and benzene exhibited good antibacterial activity with significant MICs against *E. coli*, *K. pneumoniae*, *S. aureus*, *Bacillus cereus*, *V. cholerae* and *Candida albicans* (12). Both serial and direct n-Butanol extract showed antibacterial effect only against *V. cholerae* and failed to control other seven tested microorganisms. Tania et al. (19) reported that the n-Butanol fraction from the aqueous extract of *Solidago chilensis* exhibited higher activity against *S. aureus* than the aqueous fraction of the same extract. Ethyl acetate fraction of *C. collinus* (serial) divulged remarkable antibacterial activity against almost all the tested pathogens except *L. monocytogenes* with the discrete range of zone of inhibition and MICs. Similarly Ozcelik et al. (20) also depicted the methanol extract, ethyl acetate and butanol fractions of *Crisium hypoleucum* showed activity against isolate strains of *S. aureus*. Direct ethyl acetate fraction from aqueous extract of *C. collinus* showed only low range of activity against *S. typhi*, *P. aeruginosa* and *V. cholerae* which is in consonance with the earlier finding of Suman and Elangomathavan (13). *L. monocytogenes* displayed less susceptibility to the ethyl acetate fraction. This is in concurred with the results of Adeshina et al. (21) who reported that the ethyl acetate fraction of crude methanolic leaf extract of *Alchornea cordifolia* was more effective in controlling the growth of *P. aeruginosa*, *S. aureus*, *E. coli* and the yeast *C. albicans*. Dichloromethane serial fraction of *C. collinus* exhibited antibacterial activity against only *E. coli* and *V. cholerae*, moreover other tested pathogens showed resistance. However, direct dichloromethane fraction of the *C. collinus* aqueous extract showed activity against *S. typhi*, *K. pneumoniae*, *S. aureus*, *P. aeruginosa* and *V. cholerae* furthermore other three tested pathogens showed resistance to this fraction. It is obvious that serial dichloromethane fraction has some specific phyto compounds to control *E. coli*, which might be absent or inactive form in direct fraction. On the other hand, direct dichloromethane fraction of *C. collinus* eluted significant phyto components to control pathogenic bacteria which are resistant to serial dichloromethane fraction. It is anticipated that combination of both fractions might be exploited to use as broad spectrum of bactericidal agent. Similarly dichloromethane serial fraction prepared from methanol extract of *Anthemis tinctoria* had been proven as magnificent antibacterial agent against *S. aureus* and *P. aeruginosa* (22).

The hexane fraction was found to be effective against *L. monocytogenes*, *K. pneumoniae*, *E. coli* and *V. cholerae*. However, it was ineffectual to *S. flexneri*, *S. aureus* and *S. typhi*. Direct hexane fraction showed moderate activity against *S. typhi*, *K. pneumoniae*, *E. coli*, *S. aureus* and *V. cholerae*. Both fractions failed to control *S. flexneri* and *P. aeruginosa*. The serial hexane fraction displayed maximum activity compared to aqueous extract and other fractions. Hexane fraction showed highest activity against *L. monocytogenes* and no activity in the case of *S. typhi*, this is in contrast to the antimicrobial activity of aqueous extract where *S. typhi* is sensitive and *L. monocytogenes* is resistant. Similarly Ahmed et al. (23) reported that the serial hexane fraction of four *Bauhinia* species exhibited antibacterial property against *S. aureus*, *P. aeruginosa*, *E. coli*, *Enterococcus faecalis*, *Aspergillus fumigatus*, *C. albicans* and *Cryptococcus neoformans*. The rationale behind this kind of activity may be attributed to the kind of botanicals to be released into the extract is determined by the polarity of the solvent system used. In addition, hot aqueous extract may have inactive substances which may also reduce the bacterial cidal effect of botanicals with one another (24).

The present study emphasizes that the assessment of antibacterial activity of *Cleistanthus collinus* crude aqueous extract and its fractions have given a preliminary data on the promising and non-promising solvent based fractions. Hexane fraction was found to be effective one which controls the growth of four important pathogenic bacteria. The variability in the action of the fraction against different pathogens might be attributed to the presence or absence of the specific phyto constituents available in the fractions at the given time, which is also directly depends upon the physico-chemical properties of the secondary metabolites in the solvent regime. However, a further detailed study is required to identify and characterize the active principles in each promising fraction.

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