IN-VITRO ANTIBACTERIAL SCREENING OF SWERTIA CHIRAYITA LINN. AGAINST SOME GRAM NEGATIVE PATHOGENIC STRAINS

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ABSTRACT
Antibacterial activities of aqueous and ethanolic extracts of S.chirayita was done, by an in-vitro screening through Kirby Bauer’s disk diffusion method and agar well method using CLSI guidelines against some pathogenic bacteria from clinical sources as well as from standard ATCC strains. The results were evaluated on the basis of Zone of Inhibition (in mm) compared with the standard antibiotic and Plane control i.e. the solvent used. The prepared plates were incubated at 37°C for 24 hrs. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were also evaluated. The results were analyzed statistically by using ANOVA. It was found that Chirayita is having a good antibacterial effect against some gram negative bacterial strains like E.coli, K.pneumoniae and P.vulgaris. On the similar instant, it was seen that ethanolic extract is having more efficacy as compared to the aqueous extract; it may be due to the presence of more phyto-active constituents in ethanolic extract. This study supports the traditional use of S.chirayita to cure many diseases like diarrhea, intestinal tract, throat, ear infections, fever and skin diseases. It shows that S. chirayita can be used in the infectious diseases caused by these micro-organisms. However further clinical studies are needed in this direction so that it can be used safely and effectively.

Key words: Swertia chirayita, Antibacterial activity, Zone of Inhibition, MIC, MBC

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INTRODUCTION

In this world heavily populated with bacteria, viruses and fungi, infections are the major cause of disease in human beings. Bacterial world itself is heavily populated with too many species which produce in human beings and animals, fulminating infections like tetanus, gangrene, syphilis, gonorrhea, diphtheria, leprosy, tuberculosis, urinary tract infections, respiratory tract infections etc. (Mishal and Somani, 2000).

Indiscriminate use of anti-microbial drugs cause the most infective microbes to develop resistance to many antibiotics, on the other hand infectious diseases like AIDS/HIV, anthrax, swine flu are the newer threats developed in this century, they are surging this earth and causing threat to life in present scenario, hence it become the need of an hour to search newer antibiotics, that can help in fighting the dreadful micro-organisms causing such infectious diseases. Furthermore, since the last decade the prevalence of resistance (especially multi-drug resistance) among microbial pathogens has increased astronomically. In addition, antibiotics are sometime associated with adverse effects on host like hypersensitivity, depletion of normal gut mucosal flora, immune suppression and allergic reactions. A feasible way to combat the problem of microbial resistance is the development of new antibacterial agents for substitution with ineffective ones. Thus, there is an important and necessary demand to explore the herbal flora for this, and develop new classes of effective antimicrobial agents to delay or prevent the arrival of a post-antibiotic era.

So it leads to an increase in the demand of ‘natural drugs’ which can be used against these micro-organism causing dreadful infectious diseases. Keeping all these things in mind i.e. side effects of modern antibiotics and emerging trends of development of resistance in microbes, we decided to carry out some studies to explore the use of natural drugs against some micro-organisms.

In the present study, we have selected S.chirayita, a important natural drug which is in use since ancient times for the treatment of infectious diseases like urinary tract infections, malarial fevers, respiratory tract infections as bronchitis, cough, coryza, scrofula, skin diseases, eczema, leucoderma, fevers, diarrhoea and dysentery, ulcerative colitis, vaginal infections, tumors, boils, carbuncles, inflammatory skin conditions (Rushd, 1987; Khan, 1859, Ibn-e-Sina, 1887; Ghani,1921). So, it must be having some potential to kill or inhibit the growth of micro organisms. However this property of antibiotic activity of Chirayita has not been proved scientifically so far or a very little work has been done in this field. Thus, it was decided to make a study plan to scientifically validate the claims of ancient physicians of using these drugs in infectious disease and to explore their antimicrobial activity.

MATERIAL AND METHODS

Plant collection

Swertia chirayita, the whole herb was procured from the local market Baradari of Aligarh city, U.P (INDIA) during summer (August) 2010 and was authenticated by the Department of Botany, Aligarh Muslim University, Aligarh and the botanical literature available. Sample of the test drug was kept in Advia museum, Department of Ilmul Advia, AMU, Aligarh for future references SC-0100/09-G

Crude drug Sample of Swertia chirayita

Preparation of plant extracts

The test drug was dried at room temperature in a ventilated room, milled to a fine powder and stored in a closed container in dark until use. Extraction was done according to the method described by Afaq et al., (2000) and Peach and Tracey (1955) with some minor modifications, keeping in mind that the thermo labile elements
present in the drugs are destroyed when exposed to a higher temperature beyond 55°C, so the heat wherever was needed was kept as low as possible to prevent the loss of thermolabile substances present in the drugs from destruction. Strict aseptic precautions were followed throughout the process. Aqueous extract: The coarse powdered drug were extracted using soxhlet apparatus, by reflux method with double distilled water (DDW) as a solvent at 50°C for 6 hours or until the extracting return in the siphon was colourless. The extract obtained, was subjected to dryness in the Lypholizer (Macro Scientific works, New Delhi) under reduced pressure. Ethanolic extract: The coarse powdered drug was extracted with 95% ethanol as a solvent at 50°C for 6 hours as above and dried under reduced pressure in the Lypholizer. The stock solutions for aqueous and ethanolic extract was prepared from the dried extract so obtained in the Dimethyl Sulphoxide (DMSO) as a solvent for use. The respective stock solutions so prepared were refrigerated till further use.

Test microorganisms

Bacterial strains were selected on the basis of their clinical importance in causing diseases in humans. These were obtained from different sources, clinical isolates of *E.coli*, *K.pneumoniae*, *P.aeruginosa* and *P.vulgaris* were collected from Jawaharlal Nehru Medical College & Hospital; Interdisciplinary Biotechnology Unit; Microbiology Unit, Gandhi Eye Institute, Aligarh Muslim University, Aligarh. While standard strains of the same strains *E.coli* (ATCC 26922), *K.pneumoniae* (ATCC 15380), *P.aeruginosa* (ATCC 25619), and *P.vulgaris* (ATCC 6380) were obtained from Himedia Labs Pvt. Ltd., Mumbai, India and Microbial Type Culture Collection, Chandigarh, Punjab, India.

Medium

The solid media namely Nutrient Agar No.2 (NA) (M 12695-500G, Himedia Labs Pvt. Ltd, Bombay, India) was used for preparing nutrient plates, while Nutrient Broth (NB) (M002-500G, Himedia Labs Pvt. Ltd, Bombay, India) was used for the liquid culture media.

In-Vitro Antibacterial Activity

Primary screening

Antimicrobial susceptibility testing of the aqueous and ethanolic extracts of *S.chirayita* were evaluated by Kirby Bauer’s disk diffusion (Bauer’ et al., 1969) and agar well diffusion method (Bell and Grundy, 1968). The stock solution (10 mg/ml) of the test drug was prepared by dissolving 10 mg of the herbal extract in 10 ml of Dimethyl Sulphoxide (DMSO) solvent. The stock solution was suitably diluted with sterilized distilled water to get dilution of 20 µg/ml from the stock solution. Control for each dilution was prepared by diluting 20 µl of solvent instead of stock solution with DMSO.

The bacteria were subcultured in agar medium. All the microbial cultures were adjusted to 0.5 McFarland standards, which is visually comparable to a microbial suspension of approximately 1.5 X 10^8 cfu/ml. 20 ml of agar media was poured into each Petri plate and plates were swabbed with a colony from the inoculums of the test microorganisms and kept for 15 min for adsorption. Using sterile cork borer of 6 mm diameter, wells were bored into the seeded agar plates and these were loaded with a 40µl volume with concentration of 10 mg/ml of each compound reconstituted in the dimethylsulphoxide (DMSO). All the plates were incubated at 37°C for 24 h.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial compound that will inhibit the visible growth of microorganisms after overnight incubation (Andrews, 2001). Minimum inhibitory concentrations are important in diagnostic laboratories to confirm resistance of microorganisms to antimicrobial agents and also to monitor the activity of new antimicrobial agents. The MIC of the chemically synthesized compound was tested against bacterial strains through a broth dilution method. In this method, the test concentrations of chemically synthesized compounds were made from 2.5 to 0.01 mg/ml in the sterile wells of the micro-titer plates.
**Broth Dilution Method**

In a sterile microtitre plates (96-u-shaped wells) 50 µl of the sterile nutrient broth was poured in each well in three rows, than from a fresh inoculums so formed (10^8 cfu/ml diluted with 100µl Nutrient broth to have 10^6 cfu/ml). 50 µl of the suspension was poured in each well in the first and third row, second row was again filled with 50 µl of Nutrient broth, finally the drug sample 50µl was added in the first row diluting uniformly from 2.5 to 0.01 mg/ml till the 8th well. MIC was expressed as the lowest dilution, which inhibited the growth judged by lack of turbidity in the well. All the microtitre plates were wrapped properly with a sterilized foil and incubated at 37°C for 18-24 hours.

A minimum bactericidal concentration (MBC) is the lowest concentration of antimicrobial compound that will prevent the growth of a microorganism after subculture on to antibiotic free media. Minimum bactericidal concentrations (MBCs) were determined by spreading the 100 µl extract from one below MIC and MIC itself. All the plates were incubated at 37°C for 18-24 hours and the growth was observed on each plate.

### OBSERVATION

Antimicrobial activity of all the extracts of the herbal drug was evaluated by measuring the zone of growth inhibition against the test microorganisms with Antibiotic Zone Scale (PW297, Himedia Labs Pvt. Ltd., Mumbai, India), which was holded over the back of the inverted plate. The plate was held a few inches above a black, nonreflecting background and illuminated with reflected light. The medium with dimethylsulphoxide (DMSO) as solvent was used as a negative control whereas media with Ciprofloxacin a standard antibiotic for gram Negativewas used as Negativecontrol.

Commercially prepared discs, procured from Himedia Laboratories Private limited, Mumbai, India, were used as per the Clinical Laboratory and Standard Institute (CLSI) recommendations (Anonymous, 2008). In order to clarify any effect of DMSO on the biological screening, separate studies were carried out with solutions alone of DMSO and they showed no activity against any microbial strains. The experiments were performed in triplicates.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Test strains</th>
<th>ZONE OF INHIBITION (in mm) expressed as Mean ± S.E.M (S.D) Probability of error</th>
<th>Aqueous extract</th>
<th>Ethanolic extract</th>
<th>Control (DMSO-50µl)</th>
<th>Standard (Gentamicin 30µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>E.coli</em> (clinical isolate)</td>
<td>12.4±0.60(1.34)* (S)</td>
<td>15.8±0.37(0.83) (S)</td>
<td>6.6±0.24(0.54) (R)</td>
<td>14.0±0.54(1.22) (S)</td>
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<tr>
<td>2</td>
<td><em>K.pneumoniae</em> (clinical isolate)</td>
<td>6.4±0.24(0.54)* (R)</td>
<td>19.4±0.40(0.89) (S)</td>
<td>6.6±0.24(0.54) (R)</td>
<td>14.8±0.20(0.44) (S)</td>
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<tr>
<td>3</td>
<td><em>P.aeruginosa</em> (clinical isolate)</td>
<td>6.4±0.24(0.54)* (R)</td>
<td>19.8±0.20(0.44) (S)</td>
<td>6.2±0.20(0.44) (R)</td>
<td>14.8±0.20(0.44) (S)</td>
<td></td>
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<tr>
<td>4</td>
<td><em>P.vulgaris</em> (clinical isolate)</td>
<td>6.4±0.24(0.54)* (R)</td>
<td>26.8±1.3(3.03) (S)</td>
<td>6.4±0.24(0.54) (R)</td>
<td>14.8±0.20(0.44) (S)</td>
<td></td>
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<tr>
<td>5</td>
<td><em>E.coli</em> (ATCC 25922)</td>
<td>12.4±0.60(1.34)* (S)</td>
<td>15.8±0.37(0.83) (S)</td>
<td>6.6±0.24(0.54) (R)</td>
<td>14.0±0.54(1.22) (S)</td>
<td></td>
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<tr>
<td>6</td>
<td><em>P.vulgaris</em> (ATCC 6380)</td>
<td>6.4±0.24(0.54)* (R)</td>
<td>26.8±1.3(3.03) (S)</td>
<td>6.6±0.24(0.54) (R)</td>
<td>14.8±0.20(0.44) (S)</td>
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RESULT AND DISCUSSION

Complementary and Alternative medicine is now being used worldwide. Herbal drugs are playing an important role in healthcare programs in the world. They are also showing a great role in the development of primary health care because of their effectiveness with safety and lesser side effects. The increasing incidence of infectious diseases necessitates to search for quick, effective and safe natural remedies for these health problems and this gives a search for herbal antimicrobial drug and follow the slogan of World Health Day, 2011 under their theme “Combat Drug Resistance”.

The major problem that exist with the infectious diseases and the antibiotics used for them is the emergence of antibiotic resistance. This is a worldwide problem and now the time has come when one should think about its solution from alternative therapy. Evidence based medicine is the goal for western doctors nowadays and authorities request that for any drugs used in these medicines, they should solidify their evidence on the basis of scientific background to be presented, to make their use acceptable. So, natural drugs fulfill this promise to a much extent as evident by the researches done so far. Certain antibiotics are there which have been derived from plant sources (Bhattacharjee and De, 2005).

Ethnopharmacologists, botanists, microbiologists, and natural-products chemists are combing the Earth for phytochemicals and "leads" which could be developed for treatment of infectious diseases (Cowan, 1999). An analysis of the origin of the drugs indicates that the natural products or drugs derived from natural products

Table-1 Antibacterial activity of Aqueous and Ethanolic Extracts of Chirayita Against Gram Negative Bacterial Strains

<table>
<thead>
<tr>
<th></th>
<th>P. aeruginosa (ATCC 25619)</th>
<th>E. coli</th>
<th>K. pneumoniae (ATCC 15380)</th>
<th>K. pneumoniae (ATCC 15380)</th>
<th>P. aeruginosa</th>
<th>P. vulgaris</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>6.4±0.24(0.54)* (R)</td>
<td>6.4±0.24(0.54)* (R)</td>
<td>19.8±0.20(0.44)** (S)</td>
<td>19.4±0.40(0.89)*** (S)</td>
<td>14.0±0.54(1.22) (S)</td>
<td>14.8±0.20(0.44) (S)</td>
</tr>
<tr>
<td>8</td>
<td>6.6±0.24(0.54) (R)</td>
<td>6.6±0.24(0.54) (R)</td>
<td>14.0±0.54(0.54) (R)</td>
<td>14.0±0.54(0.54) (R)</td>
<td>14.0±0.54(1.22) (S)</td>
<td>14.8±0.20(0.44) (S)</td>
</tr>
</tbody>
</table>

(S)= Sensitive to bacterial strain (R) = Resistant to bacterial strain
Significance: ***=p<0.001; **=p<0.01; *=p<0.05; ns=Not Significant

Fig. 1 MIC and MBC of The Test Drug Ethanolic Extract Against Gram Negative Bacterial Strains

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comprise of almost 30% of all new chemical entities (NCEs) launched into the market (Kaushik and Goyal, 2011). The shortcoming of the present antibiotics, propel the discovery of new pharmacotherapeutic agents in medicinal plants. Aqueous extract of the drug sample has a slightly significant inhibitory activity against E.coli ATCC 26922 (12.4±0.60; p<0.05), which was lower than inhibition produced by Gentamicin ZOI-14.8±0.20 mm. while for K.pneumoniae, P.aeruginosa and P. vulgaris it showed completely resistance.

While for the ethanolic extract of the same drug sample, it showed sensitivity to all strains and there was equal inhibitory activity towards clinical isolates or ATCC strains in the order of P.vulgaris ATCC 6380 (26.8±1.30; p<0.001) > P.aeruginosa ATCC 25619 (19.8±0.20; p<0.001) > K.pneumoniae ATCC 15380 (19.4±0.40; p<0.001)* > E.coli ATCC 26922 (15.8±0.37; p>0.05)*, All showed a significant inhibition as compared to Gentamicin (ZOI- 14.0-14.8 mm).

Whole herb of Chirayita has many benefits against several pathogenic micro-organisms. The highest antibacterial activity of Chirayita also supports the previous study done by Alam et al. (2009). They have tested various extracts and at 400µg/disk, the ZOI was 19 mm against S.aureus for dichloromethane fraction, so it reflects that Chirayita has potentiality to be used in skin infections. The activity in ethanolic extract was found to be more effective as compared to the aqueous extract. This suggests that the active principles are present in ethanol extract in high concentration / potency and not in aqueous portion. This property can be attributed to the presence of flavonoids in it as flavonoids act as an anti-oxidant and antimicrobial, they are also thought to protect plants from UV radiations and micro-organisms.

CONCLUSION

It can be concluded that among all the tested strains maximum sensitivity was that of Chirayita to six strains viz. S.aureus, S.mutans, S.epidermidis and C.xerosis. This potent antimicrobial property probably can be due to the presence of various pharmacologically active constituents in it as sterols, terpenoids and flavonoids. There is no doubt that the allopathic treatment for different diseases is quicker than the Unani system of medicine but the risk of side effects is more with the modern system of treatment. The herbs and Unani preparations have promising antimicrobial effects and can serve as complementary to the conventional treatment regimen. It has been advised that the total herb should be used instead of isolated active principle. The total herb, besides active principles, has also other constituents like resins, gum, sugar, vitamins, inorganic salts and many vegetable substances. These in-built antitodal mechanisms protect the body from the ill-effects of the herb. The medicinal plants possess some breaking or balancing mechanism, which cannot be ignored. The potent antibacterial activity so found can be the result of various active constituents present in it as they interact in complex ways to produce the needed therapeutic effect as a whole. The herbs contain hundreds and thousands of different chemical ingredients that interact in different ways, to produce the therapeutic effect.

As chirayita have shown a significant and potent antimicrobial activity. So, the present study also confirms the claims of Unani physicians who have described its use in various infectious diseases. These drugs can be used as a broad spectrum natural antimicrobial in future. The study needs further research to be carried out in this direction till its use in clinics as natural antibiotics, as this is just an in-vitro proof of its antibacterial efficacy.

REFERENCES


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