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Original Article

ANTIMICROBIAL ACTIVITY ON SEED EXTRACT OF *HIBISCUS SABDARIFFA*

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ABSTRACT

The plant *Hibiscus sabdariffa* (Malvaceae), claimed to be used traditionally in the treatment of varies ailments including rheumatoid arthritis, anti inflammatory, ant-depressent, antimicrobial and parkinsons disease etc. The *Hibiscus sabdariffa* seeds are coarsely powdered in Willy mill and successively extracted with methanol by soxhlet extraction method for 18 hrs. and concentrated under reduced pressure at 70^{o} C. For antimicrobial study, hydro alcoholic extract of *Hibiscus sabdariffa* seeds at a concentration of 50µg and 100µg per each cup exhibited considerable antimicrobial activity against tested gram +Ve bacteria like *bacillus subtilis, staphylococcus aureus* and gram –Ve bacteria like *escherichia coli* and *pseudomonas aeruginosa*. The *bacillus subtilis* and *escherichia coli* showed potent activity. *staphylococcus aureus* and *pseudomonas aeruginosa* showed moderate activity when compared to standard drug (Rifampicin).

INTRODUCTION

The plant *Hibiscus sabdariffa* (Malvaceae), claimed to be used traditionally in the treatment of various ailments including rheumatoid arthritis, antimicrobial, anti-inflammatory, anti-depressent, parkinson's disease, infertility, stomachic and emollient. Micro-organisms have proved to be beneficial to man in a number of ways and form an indispensable component of our ecosystem. Plants are known to produce a variety of compounds to protect themselves against a variety of their pathogens and therefore considered as potential source for different classes of antimicrobial substances.[1] For centuries, plants have been used in the traditional treatment of microbial infections. Plants used in traditional medicine contain a wide range of substances that can be used to treat chronic as well as acute infectious diseases. The substances that can either inhibit the growth of micro-organisms or kill them are considered as candidates for developing new drugs for treatment of various infectious diseases. [2] The use of plants in traditional system of medicine is well known in the rural areas of developing countries. [3].

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The efficacy of an antimicrobial agent can be estimated through the determination of the minimum inhibitory concentration (MIC), being the minimum concentration at which no microbial growth occurs after a specified exposure time to the antimicrobial agent.

Figure 1. *Hibiscus sabdariffa* seeds

Natural products – role as anti- microbial

Natural products have played a pivotal role in the discovery of antimicrobial drugs, with the drug either being completely derived from the natural product, or serving as a lead for novel drug discovery. From the past 6 – 7 decades most of the antimicrobials have been discovered through screening of soil samples, of which the antimicrobial efficacies were determined first *in vitro* and later *in vivo*. There are plenty of examples of such naturally occurring antimicrobials and include drug classes such as the penicillins and cephalosporins (lactam being the empirically active component), all of which were discov-

ered in or derived from fungi. Numerous other antimicrobials were derived from different filamentous strains of the bacterial genus *Streptomyces*, including streptomycin, erythromycin, tetracycline and vancomycin. Semi-synthetic modifications to these naturally-occurring drugs have brought about the production of second and third generation lactams of both the penicillin and cephalosporin classes, while complete synthesis produced yet more active compounds, specifically the second-generation erythromycins, viz. clarithromycin and azithromycin. As of the end of 1999 only the fluoroquinolones represent a totally synthetic, significant class of antibiotics. [4] As majority of the world's plant species are not yet explored in this regard, the exploitation of the medicinal potential of these species will prove to be both interesting and challenging to scientists from diverse fields of expertise. Plants synthesize a diverse array of secondary metabolites, which play a key role in the natural defense mechanisms. Antimicrobial phytochemicals [12] are now divided into different chemical categories: phenolics, terpenoids and essential oils, alkaloids, lectins and polypeptides, as well as polyacetylene. An increase in the isolation and identification of such compounds may thus contribute greatly to the success in antibiotic discovery. Antimicrobial activity of plants can be detected by observing the growth response of various microorganisms to those plant tissues or extracts, which are placed in contact with them. There are various methods for detecting antimicrobial activity but results obtained will also be influenced by the method selected and the microorganisms used for testing. To detect antimicrobial activity of plant extracts, three conditions must be fulfilled. First, the plant extract must be brought into contact with the cell wall of the microorganisms that have been selected for the test. Second, conditions should be provided such that the microorganisms are able to grow when no antimicrobial agents are present. Third, there must be some means of judging the amount of growth, if any made by the test organisms during the period of time chosen for the test. Initial screening for potential antibacterial activity may be performed with pure substances or crude extracts.[5] In order to detect the antimicrobial substances present in very small quantities in plant extract, testing is carried out on the extracts in the form in which they are prepared or on concentrated extracts. The water insolubility of lipophilic samples (non-polar extracts) makes it necessary to use other solvents such as alcohol, acetone, chloroform, dimethyl sulphoxide, dioxane, glycerol and different emulsifiers such as macrogol ethers, sorbitan and cellulose derivatives. The solvent used for diluting the sample should be inert ie. It should not have any antimicrobial activity. The pH of the samples should be checked before testing ; the extracts are best adjusted to pH between 6.0-8.0 or dissolved in buffer solution such as physiological Tris buffer or others.

MATERIAL AND METHODS

Plant material collection and authentication[1]

The plant material of *Hibiscus sabdariffa* was collected Annapurna agro agencies, sabbavaram, Andhra Pradesh, India in December 2014. The plant species was authenticated by Dr. Bodaih Padal, taxonomist, department of botany, Andhra university, Visakhapatnam. The voucher specimens (21921) were deposited in the herbarium, college of pharmaceutical sciences, Andhra university.

Soxhlet Extraction Method

The dried coarsely powdered materials of seeds were extracted sucessively three times with methanol by soxhlet extraction method. The obtained extract were concentrated and dried completely, weighed and stored in a dessicator

Methods for evaluating antimicrobial activity [6]

The term microbiological assay is a biological assay performed with micro-organisms like bacteria, yeast, moulds etc. This involves the measurement of the relative potency or activity of compounds by determining the amount of test material required for producing stipulated effect on suitable organism under standard conditions.

The procedures employed in microbial assay were,

a) Cylinder plate method or cup plate method

b) Turbidometric or tube assay method (two fold serial dilution method).

In the present study, antimicrobial screening was carried out using cup plate method

In cup plate method, the antimicrobial [7] substance diffuses from the cup through a solidified agar layer in a Petri dish or a plate to some extent so that the growth of added micro-organism is inhibited entirely in acircular area or zone around the cavity containing the solution of a known quantity of antimicrobial substance. The antimicrobial activity [8] is expressed as the zone of inhibition in millimeters, which is measured with a zone reader. The methanolic extracts of *Hibiscus sabdariffa* were screened for antimicrobial activity against a wide spectrum of microorganisms and the activity was compared with appropriate reference standards (chloramphenicol for both gram-positive and gram-negative organisms). Micro-organisms were grown in nutrient agar medium. Dimethyl sulphoxide (DMSO) and distilled water were used as control and the drug vehicles for the plant extracts and reference standards respectively.

Table 1. Antimicrobial activity

Dose	Zone of inhibition(mm) Gram positive		Zone of inhibition(mm) Gram negative	
	B.S	**S.A**	**E.C**	**P.A**
Extract (50µg/ml)	13	08	12	08
Extract (100µg/ml)	18	11	15	11
Standard (Rifampicillin)	24	22	19	24
Control (methanol)	0.9			

S.A= Staphylococcus aureus ; B.S= Bacillus subtilis ; E.C= Escherichia coli; S.A= Pseudomonas aeruginosa

Test Organisms:

The microorganisms used for the experiments were procured from MTCC, IMTECH, Chandighar.

Gram-positive organisms:

Staphylococcus aureus and *Bacillus subtilis.*

Gram-negative organisms:

Escherichia coli and *Pseudomonas aeruginosa*.

Standardization of micro-organisms [1]

One loop-full of micro-organisms were inoculated into 100 ml of sterile medium and incubated for 24 h at 37°c for bacterial culture and for 48 h at 27°c for fungal culture. After 24 h/48 h of incubation, 1 ml of broth containing the micro-organisms was added to 9 ml of peptone water. 10 fold serial dilutions were made in the range of 10^{-1} to 10^{-10}. 100 µl of the dilutions ranging from 10^{-5} to 10^{-8} were spread over the sterile nutrient agar (SDA) plates and kept at 37 and 27°c for 24 / 48 hours respectively. The number of colony forming units (CFU) was counted and number of micro-organisms per 1 ml of stock culture was calculated.

Preparation of test and standard solutions

The stock solution of test compounds was prepared by dissolving the dried extracts at a concentration of 5 and 10mg/ml in dimethylsulphoxide (DMSO) respectively. The stock solution of reference standards (rifampicillin) was prepared at a concentration of 0.6 mg/ml in sterile water. Antimicrobial activity was screened by adding 0.05 ml stock solution to each cup by micropipette.

Culture medium:

The following media were used for the present antimicrobial studies.

Nutrient broth for bacteria

Beef extract - 0.35%,

Sodium chloride - - 0.5%

Peptone - 0.5%

The above ingredients weighing 37 g were dissolved in distilled water (1000 ml). pH was adjusted to 7.2-7.4 and sterilized by autoclaving at 15 lbs for 20 min.

Sterilization

Sterilization of the media, water etc., were carried out by autoclaving at 15 lbs/inch^2 for 20 minutes. The glassware like syringes, petri dishes, pipettes, empty test-tubes were sterilized by dry heat in an oven at a temperature of 160°c for one hour.

Nutrient agar for bacteria

Beef extract-0.3%, Sodium chloride -0.5%, Peptone-0.5%, Agar-2.0% and pH-7.2-7.4

The sterilized medium was cooled to 40°c and poured into the Petri dishes to contain 6 mm thickness. The media was allowed to solidify at room temperature.

Evaluation of antibacterial activity

Determination of zone of inhibition by cup plate method [9-11]

The cylinder plate assay of drug potency is based on measurement of the diameter of zone of inhibition of microbial growth surrounding cylinders (cups), containing various dilutions of test compounds.A sterile borer was used to prepare four cups of 6 mm diameter in the agar medium spread with the micro-organisms and 0.1 ml of inoculum. These cups were spread on the agar plate by spread plate technique. Accurately measured (0.05 ml) solution of each concentration and reference standards were added to the cups with a micropipette.

All the plates were kept in refrigerator for 2 hours for effective diffusion of test compounds and standards. Later, they were incubated at 37°C for 24 hours. The presence of definite zone of inhibition of any size around the cup indicated antibacterial activity.The solvent control was run simultaneously to assess the activity of dimethyl sulphoxide and water which were used as a vehicle. The experiments were performed three times. The diameter of the zone of inhibition was measured and recorded.

RESULTS

All extracts of selected plant at a concentrations of 50 µg and 100 µg per each cup exhibited considerable antibacterial activity against tested bacterial species (gram +ve and gram –ve). The *bacillus subtilis* showed potent activity when compared to *staphylococcus aureus.* And for Gram negative *Escherichia*

coli showed moderate activity when compared to *Pseudomonas aeruginosa.* All extracts showed better anti-bacterial activity against gram +ve bacteria than gram -ve bacteria. The results of antibacterial activity are shown below.

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