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EVALUATION OF ANTIMICROBIAL ACTIVITY OF DIFFERENT SOLVENT EXTRACTS OF FRUITS OF MEDICINAL PLANT: *ZANTHOXYULUM RHETSA* ROXB DC.

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ABSTRACT

Antimicrobial efficiency of various solvent extracts of the fruits (carpel and seed) of *Zanthoxyulum rhetsa* Roxb DC a medicinal plants of the Ruteacea family collected from the Western Ghat region were tested *Escherichia coli, Salmonella typhi, Bacillus cereus, Bacillus subtilis, Methicillin-resistant Staphylococcus aureus, Staphylococcus aureus, Enterococcus faecalis, Klebsiella aerogenes, Shigella flexneri, Pseudomonas aeruginosa, Aspergillus niger Candida albicans, Aspergillus falvus and Rhizopus oryzae.* Both the carpels and seeds extract Methanolic, Petroleum ether, Ethyl acetate, Chloroform, Ethanolic extracts of showed significant activity against *Escherichia coli, Salmonella typhi, Salmonella aureus, Enterococcus faecalis, Klebisella aerogenes, Pseudomononas aeruginosa, Aspergillus niger, Candida albicans* wheras aqueous extract showed no antimicrobial activity against all the experimental strains. The strongest antibacterial activity observed for the Methanol extracts of Seeds against *S. aureus & S. typhi,* Chloroform extract of seeds against *S. typhi* and Ethyl acetate extracts of carpels *S. aureus.* The strongest antifungal activity observed for the Chloroform extract of carpels against *C. albicans* and Petroleum extracts of seeds against *A. niger.* The Spectrum of activity observed in the present study may be indicative of the present study extracts of the fruits of *Zanthoxyulum rhetsa* Roxb DC could be a possible source to obtain new and effective herbal medicines to treat infections, hence justified the ethnic uses of *Zanthoxyulum rhetsa* Roxb DC against various infectious diseases.

KEYWORDS: Antimicrobial activity, *Zanthoxyulum rhetsa* Roxb DC., medicinal plants, Agar well diffusion method.

INTRODUCTION

Antibiotics produced by a number of pharmacological industries are one of our most important weapons in fighting microbial infections and improving the quality of treatment. An antimicrobial activity of a plant is a compound that kills or inhibits the growth of microbes such as bacteria and fungi. (Jagessar, R.C et.al. 2008). Between 1981 and 2002 about 61% of new drugs developed were based on plant products and have been successful, especially in the areas of infectious disease and cancer (Cragg GM, Newman DJ, 2005). Recently in the past few decades, the commonly used antibiotics have become less effective against certain illnesses due to their toxic reactions and also due to drug-resistant bacteria. It is very important to investigate newer drugs with lesser resistance. Drugs which are derived from natural sources play a significant role in the prevention and treatment of various human diseases. (Houghton PJ, 1995, Farnsworth NR, 1993).Man has used medicinal plants to treat common infectious diseases and some of these traditional medicines are still being included as part of the habitual treatment of various maladies. Medicinal plants are a rich source of antimicrobial agents as they possess secondary metabolites which can combat disease causing pathogens. (Karinge, J.W., 2006, Bandaranayake, M., et.al. 2006, Mosihuzzanman, M et.al. 2008). A number of medicinal plant parts are used for extract as raw drugs and possess varied medicinal properties to cure various diseases. (Gisesa, W.N.O., 2004, Egwaikhide, P.A.2007). Primitive people learned by trial and error method to distinguish useful plants with beneficial effects. (Jagessar, R.C et.al. 2008). In spite of great advances in modern medicine in recent decades, plants still make a very important contribution to health care (Ravikumar, S., 2010). A number of factors such as preference of consumers for natural therapies, the belief that herbal medicines might be more effective and are free from side effects where the conventional therapies have proven to be inadequate; improvement in quality proof of efficacy and safety of herbal medicines and its cost in comparison to synthetic medicines have contributed to the growth of the use of traditional herbs worldwide ,(Ravikumar, S.,2010, Ceylan, E, 2004, De, A.K., 2004, Rattanachaikunsopon, P, 2010, Jagessar, R.C.,2007). Consequently, it is very important for researchers to find out the particular micro-organisms for which the herbal extracts are active. (Ravikumar, S., 2010). The objective of this study was to determine the antibacterial effect of various extract of seeds and carpels against thirteen pathogens like Bacteria: : *Escherichia coli* , *Salmonella*

typhi, Bacillus cereus, Bacillus subtilis, Methicillin-resistant Staphylococcus aureus (MRSA), *Staphylococcus aureus, Enterococcus faecalis, Klebsiella aerogenes, Shigella flexneri, Pseudomonas aeruginosa* and fungi cultures : *Aspergillus niger, Candida albicans, Aspergillus falvus and Rhizopus oryzae* using agar well diffusion.

Zanthoxyulum rhetsa Roxb DC (Ruteacea) has the potential to determine authenticity and reliability of chemical constituent of herbal drug and formulation.

MATERIALS AND METHODS

Study area

The carpels and seeds of *Zanthoxyulum rhetsa R*oxb DC was collected in 2012, 2013 from the Western Ghats.

Materials used in the research were autoclaved. Glass rods and loops were disinfected by dipping in alcohol and then flaming on a Bunsen burner, Laminar flow and biosafety lab. The working bench was swabbed with 70% alcohol before and after each experiment. Experimental work was carried out in Haffkine Bacteriology department.

Preparation of plant material

Fresh carpels and seeds were washed thoroughly with water and then air dried at room temperature for five days. After drying, the fruits were opened and the carpels and seeds were separated and were ground into powder and then sieved using a sieve. Two kilograms of powdered plant extracts were transferred into airtight containers and stored at room temperature.

Microorganisms Collection

The cultures of *Escherichia coli , Salmonella typhi, Bacillus cereus, Bacillus subtilis, Methicillin-resistant Staphylococcus aureus, Staphylococcus aureus, Enterococcus faecalis, Klebsiella aerogenes, Shigella flexneri, Pseudomonas aeruginosa, Aspergillus niger Candida albicans, Aspergillus falvus and Rhizopus oryzae* were obtained from Bacteriology department, Haffkine Institute. (Table 1)

Standards Discs used were obtained from HIMEDIA (Table 1)

Table 1: List of Microorganisms with ATCC/ NTCC and Standards Disc used against Microorganisms

Name of Microorganism (Bacterial)	ATCC/NCTC	Standard
Escherichia coli	ATCC 25922	Azetronam
Salmonella typhi	NCTC 786	Erythromycin
Bacillus cereus	ATCC 11778	Gentamincin
Bacillus subtilis	ATCC 9372	Gentamincin
Methicillin-resistant Staphylococcus aureus	ATCC 25923	Vancomycin
Staphylococcus aureus	ATCC 6538	Amoxyclav
Enterococcus faecalis	ATCC 29212	Co- Trimoxazole
Klebsiella aerogenes	Clinical Isolate	Piperacillin
Shigella flexneri	Clinical Isolate	Ampicillin
Pseudomonas aerogenes	ATCC 1542	Piperacillin

Extraction of the crude extracts from carpel and seeds powder.

The active components of the carpels and seeds were extracted using the cold extraction method (Farnsworth, N.R. 1988). Six different extraction solvents namely methanol, ethyl acetate, chloroform, ethanol, Petroleum Ether and distilled water were used respectively.

To 500ml each of pure methanol, ethyl acetate, chloroform, ethanol, Petroleum Ether and sterile distilled water were added 50g portions of the carpels and seeds in sterile conical flasks and allowed to soak at room temperature for 48 hours. And further on a cold shaker for 72 hours at 120 rpm was used to improve extraction of various phyto-chemicals. The filtrate was obtained by means of a vacuum filter pump through a 127c-1 filter funnel aided by a Whatman filter paper. Filtering was repeated thrice until the solution was clear. The filtrate was evaporated in a weighed procelian dish on a water bath. Drying was done to allow the calculation of the yield of the extraction process. The extraction efficiency was quantified by determining the weight of each of the extracts and the percentage yield was calculated as (weight of dry extracts in grams /initial dry plant extracts) × 100. The procedure was done separately for the six solvents used. A small proportion of dry extracts was stored for phyto-chemical analysis. For the preparation of dilutions of dry extracts for antibacterial assay, dry extracts were reconstituted by re-dissolving in $DMSO_4$ solvent. The final filtrates were filter-sterilized by using Whatman's Filter paper. Sterile extracts obtained were stored separately in labelled, sterile capped bottles, (Table 2) in a refrigerator at 4°C before use during the antimicrobial activity.

Table 2: Extract type

S.No.	Type of Extract
1	Aqueous extract of carpel of *Zanthoxyulum rhetsa R*oxb DC
2	Aqueous extract of seed of *Zanthoxyulum rhetsa R*oxb DC
3	Ethanolic extract of carpel of *Zanthoxyulum rhetsa R*oxb DC
4	Ethanolic extract of seed of *Zanthoxyulum rhetsa R*oxb DC
5	Methanolic extract of carpel of *Zanthoxyulum rhetsa R*oxb DC
6	Methanolic extract of seed of *Zanthoxyulum rhetsa R*oxb DC
7	Ethyl acetate extract of carpel of *Zanthoxyulum rhetsa R*oxb DC
8	Ethyl acetate extract of seed of *Zanthoxyulum rhetsa R*oxb DC
9	Petroleum extract of carpel of *Zanthoxyulum rhetsa R*oxb DC
10	Petroleum extract of seed of *Zanthoxyulum rhetsa R*oxb DC
11	Chloroform extract of carpel of *Zanthoxyulum rhetsa R*oxb DC
12	Chloroform extract of seed of *Zanthoxyulum rhetsa R*oxb DC

Microbial Screening

Antimicrobial activities of thirteen different extracts of carpels and seeds of *Zanthoxyulum rhetsa R*oxb DC were evaluated by the agar well diffusion method (Murray et al.1995) modified by (Olurinola, 1996).

Media Preparation and its sterilization

Agar well diffusion method (Murray et al, 1995) later modified by (Olurinola 1996) antimicrobial susceptibility was tested on solid (agar-agar) media in petri plates. For bacterial assay nutrient agar (NA) (40 gm/L) and for fungus PDA (39 gm/L) was used for developing surface colony growth. The suspension culture for bacterial cell growth was done in Nutrient broth and for fungal cells in PDB % (w/v) PDB (Potato dextrose broth) was taken for evaluation. All the media prepared was then sterilized by autoclaving the media at (121°C) for 20 min.

Agar well diffusion method

Agar well-diffusion method was followed to determine the antimicrobial activity. Nutrient agar (NA) and Potato Dextrose Agar (PDA) plates were swabbed (sterile cotton swabs) with 8 hour old -broth culture of respective bacteria and fungi. Wells (10mm diameter and about 2 cm a part) were made in each of these plates using sterile cork borer. Stock solution of each plant extract was prepared at a concentration of 1 mg/ml in different plant extracts viz. Methanol, Ethanol, Petroleum Ether, and Water. About 100 µl of different concentrations of plant solvent extracts were added sterile syringe into the wells and allowed to diffuse at room temperature for 2hrs. Control experiments comprising inoculums without plant extract were set up. The plates were incubated at 37°C for 18-24 hours for bacterial pathogens and 28°C

for 48 hours fungal pathogens. The diameter of the inhibition zone (mm) was measured and the activity index was also calculated. Triplicates were maintained and the experiment was repeated thrice, for each replicates the readings were taken in three different fixed directions and the average values were recorded.

Test for antimicrobial activity

The antibacterial assay was carried out by micro dilution method in order to determine the antibacterial activity of compounds tested against the pathogenic bacteria. The bacterial suspensions were adjusted with sterile saline to a concentration of 1.0 X 10^7 CFU/ml. The inocula were prepared and stored at 4^o C until use. Dilutions of the inocula were cultured on solid medium to verify the absence of contamination and to check the validity of the inoculum. All experiments were performed in duplicate and repeated three times.

Test for antifungal activity

In order to investigate the antifungal activity of the extracts, a modified micro dilution technique was used. The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0 –10^7in a final volume of 100µl per well. The inocula were stored at 4^o C for further use. Dilutions of the inocula were cultured on solid potato dextrose agar to verify the absence of contamination and to check the validity of the inoculums.

OBSERVATION AND RESULT

In the present investigation, the inhibitory effect of different extracts of in vivo carpels and seeds from *Zanthoxyulum rhetsa R*oxb DC were evaluated against both fungicidal and bacterial strains.

The antimicrobial activity was determined using agar well diffusion method summarized in Table 3 & 4. The activity was quantitatively assessed on the basis of inhibition zone and their activity index was also calculated. Measurement of antimicrobial activity using Agar well diffusion.

RESULTS AND DISCUSSION

A total of 12 extracts of Carpel and Seeds were tested for antibacterial activity qualitatively as well as quantitatively against the 10-bacterial species and 3 fungi species.

1). Although some extracts exhibited a good antibacterial activity towards different tested bacterial isolates, many plant extracts exhibited a limited antibacterial activity against the test bacterial isolates (Table 4 & 5). The strongest antibacterial activity observed for the Methanol extracts of Seeds against *S. aureus* (18mm), *S. typhi* (18mm), *E. faecalis* (15mm) followed by Petroleum extracts of seeds *E. coli (15mm), E. faecalis* (13mm) *A. niger* (18 mm) and *C. albicans (17mm),* Chloroform extract of seeds *E. coli (13mm), S. typhi* (18mm) and *S. aureus* (16mm) Ethyl acetate extracts of seeds *E. coli (15mm), K. aerogenes* (15mm) and *P. aeruginosa* (15mm). (Graph 1)

2) The strongest antibacterial activity observed for the Chloroform extract of carpels *A. niger* (16 mm) and *C. albicans (32mm),* followed by Petroleum extracts of carpels *E. coli (14mm), S. typhi* (15mm) *S. aureus* (14mm) *E.* f*aecalis.* (11mm), A. *niger* (21mm) and *C. albicans (17mm),* Ethanol extracts of carpels against *S. aureus* (14mm), *E. faecalis* (11mm), Ethyl acetate extracts of carpels *S. aureus* (19mm) and *E. faecalis* (12mm) *and* Methanol extracts of carpels against *S. aureus* (13mm) and *E. faecalis* (11mm) (Graph 2)

Table 3: Antimicrobial activity of Seeds of *Zanthoxyulum rhetsa Roxb* DC.

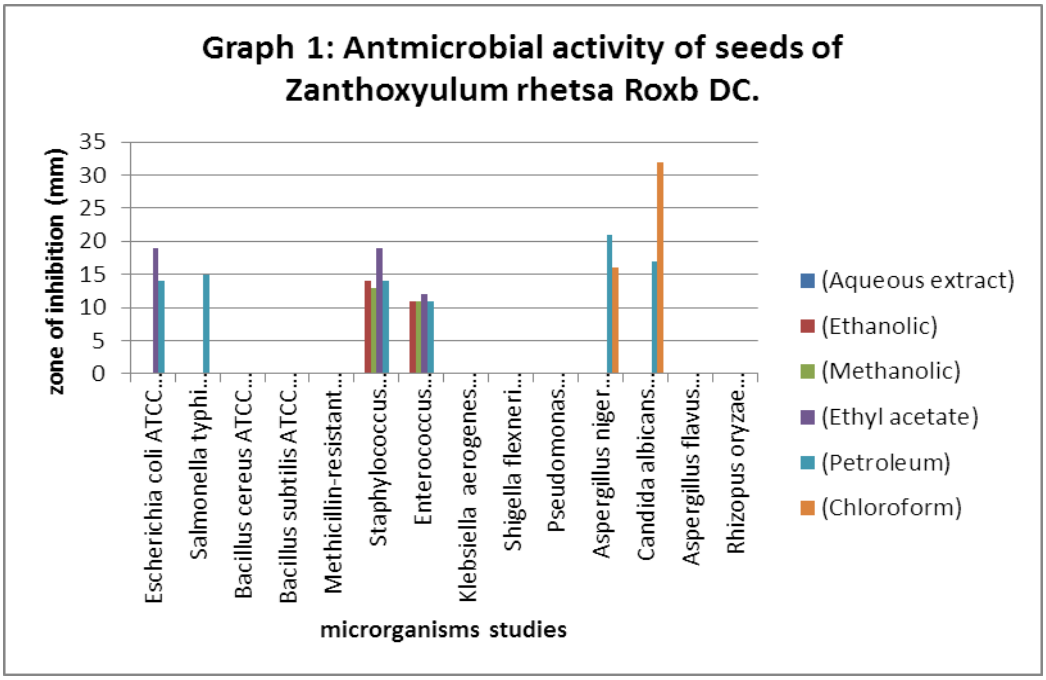
Microorganism bacteria	**2 (Aqueous extract)**	**4 (Ethanolic)**	**6 (Methanolic)**	**8 (Ethyl acetate)**	**10 (Petroleum)**	**12 (Chloroform)**
Escherichia coli ATCC 25922	Nil	Nil	Nil	15mm	15mm	13mm
Salmonella typhi NCTC 786	Nil	Nil	18mm	Nil	Nil	11mm
Bacillus cereus ATCC 11778	Nil	Nil	Nil	Nil	Nil	Nil
Bacillus subtilis ATCC 9372	Nil	Nil	Nil	Nil	Nil	Nil
Methicillin-resistant Staphylococcus aureus (MRSA) ATCC 25923	Nil	Nil	Nil	Nil	Nil	Nil
Staphylococcus aureus ATCC 6538	Nil	Nil	18mm	Nil	Nil	16mm
Enterococcus faecalis ATCC 29212	Nil	10mm	15mm	Nil	13mm	Nil
Klebsiella aerogenes	Nil	Nil	Nil	15mm	Nil	Nil

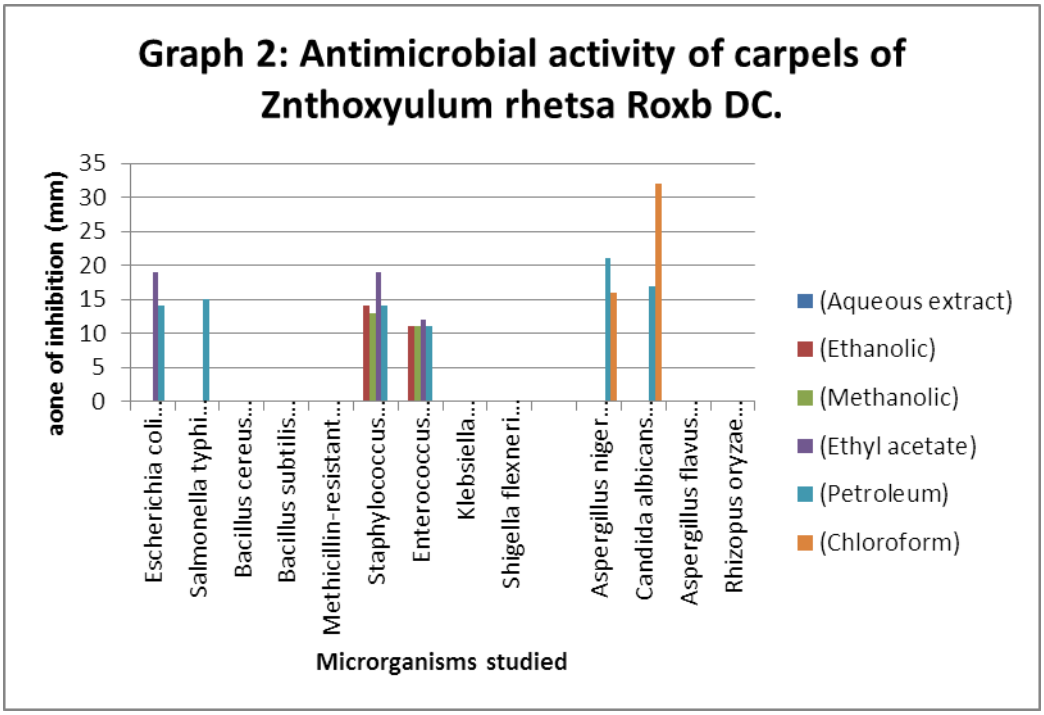
Clinical isolate						
Shigella flexneri Clinical isolate	Nil	Nil	Nil	Nil	Nil	Nil
Pseudomonas aeruginosa ATCC 1542	Nil	Nil	Nil	15mm	Nil	Nil
Aspergillus niger	Nil	Nil	Nil	Nil	18mm	Nil
Candida albicans	Nil	Nil	Nil	Nil	17mm	Nil
Aspergillus falvus	Nil	Nil	Nil	Nil	Nil	Nil
Rhizopus oryzae	Nil	Nil	Nil	Nil	Nil	Nil

Table 4: Antimicrobial activity of Carpels of *Zanthoxyulum rhetsa Roxb* DC

Name of the organism	**1 (Aqueous extract)**	**3 (Ethanolic)**	**5 (Methanolic)**	**7 (Ethyl acetate)**	**9 (Petroleum)**	**11 (Chloroform)**
Escherichia coli ATCC 25922	Nil	Nil	Nil	19mm	14mm	Nil
Salmonella typhi NCTC 786	Nil	Nil	Nil	Nil	15mm	Nil
Bacillus cereus ATCC 11778	Nil	Nil	Nil	Nil	Nil	Nil
Bacillus subtilis ATCC 9372	Nil	Nil	Nil	Nil	Nil	Nil
Methicillin-resistant Staphylococcus aureus ATCC 25923	Nil	Nil	Nil	Nil	Nil	Nil
Staphylococcus aureus ATCC 6538	Nil	14mm	13mm	19mm	14mm	Nil
Enterococcus faecalis ATCC 29212	Nil	11mm	11mm	12mm	11mm	Nil
Klebsiella aerogenes Clinical isolate	Nil	Nil	Nil	Nil	Nil	Nil
Shigella flexneri Clinical isolate	Nil	Nil	Nil	Nil	Nil	Nil
Pseudomonas aeruginosa ATCC 1542	Nil	Nil	Nil	Nil	Nil	Nil
Aspergillus niger	Nil	Nil	Nil	Nil	21mm	16mm
Candida	Nil	Nil	Nil	Nil	17mm	32mm

albicans						
Aspergillus falvus	Nil	Nil	Nil	Nil	Nil	Nil
Rhizopus oryzae	Nil	Nil	Nil	Nil	Nil	Nil





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