



ANTIMICROBIAL ACTIVITY OF EXTRACTS OF *ZANTHOXYLUM RHETSA* (ROXB.) DC. AGAINST FOOD PATHOGENS

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ABSTRACT

The pericarps and seeds of *Zanthoxylum rhetsa* were collected and powdered. The powdered material was subjected to kinetic maceration and soxhlet extraction with methanol, dichloromethane, ethyl acetate and n-hexane to get respective extracts. The extracts of pericarps and seeds were screened for its antimicrobial activity using agar cup method. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values were also determined. *Zanthoxylum rhetsa* exhibited narrow spectrum antimicrobial activity with MIC value ranging between 0.312 mg/ml to 12.5 mg/ml. It can be concluded from the antimicrobial data that *Z. rhetsa* has promising antimicrobial activity against the food pathogens; hence it may be considered as a safe antimicrobial agent.

Keywords: *Zanthoxylum rhetsa*, Agar cup, MIC, MBC

1. INTRODUCTION

The rising spate of food borne diseases caused by microorganisms is a serious health issue all over the world. To add to the woes, there is antibiotic resistance of some pathogens that are associated with foodborne illness. Hence, it is necessary to control the pathogens which may significantly reduce the food borne disease outbreaks [1]. Chemical preservatives are usually used to prevent the growth of pathogenic and spoilage microorganisms but they have detrimental effects such as carcinogenicity, teratogenicity and residual toxicity. The growing concern about food safety has led to the development of natural antimicrobials [2]. Plant-derived antimicrobials have an advantage over the synthetic drugs because they have lesser side effects [3]. Despite their tremendous therapeutic potential, this gigantic source of medicines is untapped and thus, need to be explored further in the search of novel antimicrobials.

Spices are well known to impart flavour and colour to foods and as an appetizer. They also have preservative and medicinal properties and are used since ages. It is well documented that they extend the shelf life of foods as well as prevent food spoilage and deterioration [4]. Herbs and spices are rich in secondary metabolites which are the active ingredients of plants against microorganisms [5].

Zanthoxylum rhetsa belongs to the family Rutaceae and it has been used in traditional medicinal system for centuries [6]. The secondary metabolites of the genus *Zanthoxylum* include lignoids, xantholectin, sesamin, essential oils, alkaloids, amides, flavonoids, terpenes, steroids and coumarins [7]. There are few reports on antimicrobial activity of *Z. rhetsa* pericarp and seed extracts against food pathogens.

Thus, this study was undertaken to assess the *in vitro* antimicrobial activity of crude extracts of the pericarp and seeds of *Zanthoxylum rhetsa* against selected four food-associated bacteria, namely, *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 11778, *Salmonella abony* ATCC 6017 and *Shigella boydii* ATCC 8700. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the extracts was also determined.

2. MATERIAL AND METHODS

2.1. Plant material

Zanthoxylum rhetsa plant fruits was collected from APMC market in Vashi, Navi Mumbai. The pericarps and seeds were powdered separately and used for the preparation of different solvent extracts.

2.2. Extraction methods

The pericarps and seeds were extracted in the organic solvents methanol, dichloromethane, ethyl acetate and n-hexane using kinetic maceration and soxhlet extraction.

The extracts were then filtered with Whatman No.1 filter paper. For microbiology assay, the organic solvents after extraction were evaporated and the dried extracts were dissolved in Dimethyl Sulfoxide (DMSO) [8].

2.3. Antimicrobial activity

2.3.1. Culture and maintenance of microorganisms

The food-borne bacteria chosen for this assay were *Staphylococcus aureus* (*S. aureus*) ATCC 25923, *Bacillus cereus* (*B. cereus*) ATCC 11778, *Salmonella abony* (*S. abony*) ATCC 6017 and *Shigella boydii* (*S. boydii*) ATCC 8700. All these bacterial cultures were obtained from Guru Nanak Institute of Research and Development (GNIRD) and Microbiology Department of G. N. Khalsa College, Matunga. They were maintained on nutrient agar medium at 4°C till further use.

2.3.2. Inoculum preparation

A single colony was inoculated in 50 ml of sterile Mueller Hinton broth and incubated at 37°C for 24 hours. The absorbance of bacterial cells was adjusted to 0.1 at 620 nm corresponding to 1×10^8 CFU/ml for the antibacterial assay [9].

2.3.3. Agar well diffusion

Antimicrobial activities of all the kinetic macerated and soxhlet extracts of pericarps and seeds of *Z. rhetsa* were carried out using the agar well diffusion method following the guidelines provided by Clinical Laboratory Standards Institute (CLSI) [10].

Mueller-Hinton agar medium (MHA) was used for antimicrobial susceptibility tests. Petri plates containing 20 ml MHA was seeded with bacterial strains. After solidification of agar, equidistant wells (8 mm diameter) were made by cork borer. Fifty µl of respective plant extracts were loaded in the respective wells. Plates were incubated at 37°C for 24 hours and zone of inhibition was measured in mm. The individual solvents (Methanol, Dichloromethane, Ethyl Acetate, n-hexane) were used as negative control [9, 11]. Ciprofloxacin (5 ppm) served as the positive control [12]. All experiments were repeated three times independently.

2.3.4. Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of the antimicrobial agent that will inhibit the visible growth of a microorganism after overnight incubation [13]. Two-fold microdilution broth method was used to determinate the MIC value [14].

A stock solution of the plant extracts of *Zanthoxylum rhetsa* was prepared and serially diluted in 96-wells microtiter plate with Mueller Hinton broth to obtain a concentration of 25, 12.5, 6.25, 3.12, 1.56, 0.78 mg/ml. A standardized inoculum for each bacterial strain was prepared to give an inoculum size of approximately 5×10^5 CFU/ml in each well. Two controls were maintained for each test batch. Positive control well contained inoculated growth medium without test samples. The negative control well included uninoculated medium and DMSO. Microtiter plates were then kept at 37°C for an overnight incubation. The MIC of each extract was determined post-incubation by adding 40 µl p-iodonitrotetrazolium salt (INT dye) and further incubating at room temperature for 30 minutes. Viable bacteria reduced this yellow dye to pink. The MIC of each sample was defined as the lowest concentration that prevented this change and resulted in the complete inhibition of microbial growth [15].

2.3.5. Determination of minimum bactericidal concentration (MBC)

Bactericidal Concentration (MBC) was determined by streaking a loopful of the sample from the wells with concentrations of MIC and above the MIC on fresh Mueller Hinton Agar plates. The MBC was determined as the lowest concentration of the extract after sub-culturing which led to no bacterial growth, indicating 99.5% killing of the original inoculums [16].

2.3.6. Statistical analysis

All the data were reported as mean $\pm$ standard deviation of three replicates. Statistical analysis was performed using Microsoft Excel.

3. RESULTS AND DISCUSSION

3.1. Antimicrobial activity

Preliminary assessment of antibacterial activity of the extracts was primarily done using Agar well diffusion technique and subsequent quantification was carried out by Minimum Inhibitory concentration (MIC), Minimum Bactericidal Concentration (MBC) and ratio of MBC and MIC. The pictorial results indicating the antimicrobial activity of all the kinetic macerated and soxhlet extracts of pericarps and seeds against *S. aureus* and *B. cereus* are presented in Fig.1-2. The zone of inhibition values against all the pathogenic microorganisms are enlisted in Table 1-2. The quantification results can be observed in Table 3-4.

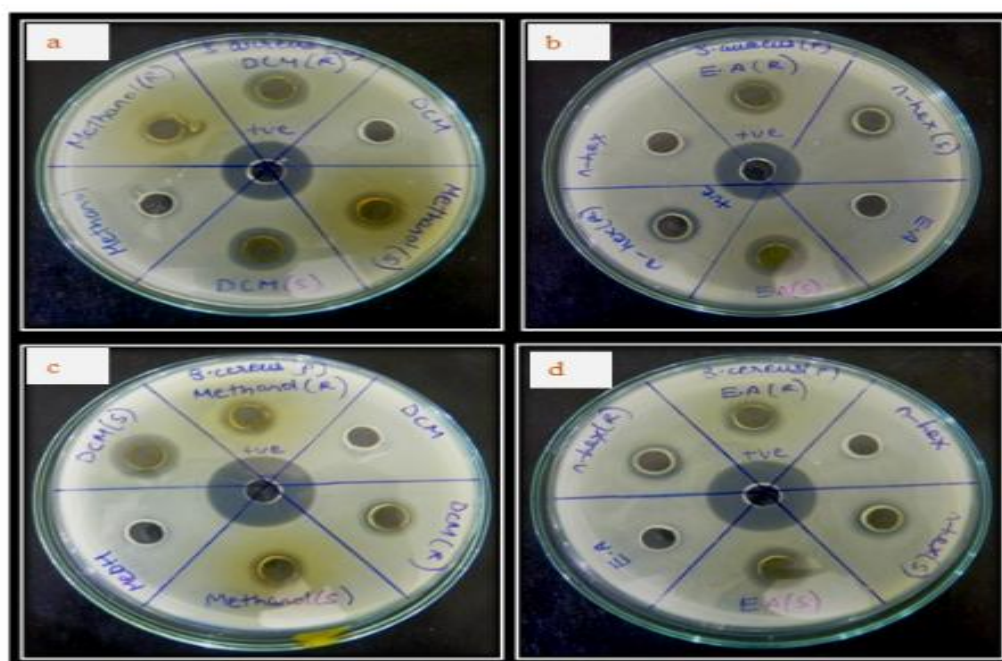


Fig. 1(a, b): Antimicrobial Activity of *Z.rhetsa* Pericarp (P) extracts against *S. aureus*, (c, d): Antimicrobial Activity of *Z.rhetsa* Pericarp (P) extracts against *B.cereus*

Table 1: Zone of inhibition of *Zanthoxylum rhetsa* pericarp extracts (mm)

<i>Zanthoxylum rhetsa</i> Pericarp	<i>Staphylococcus aureus</i>	<i>Bacillus cereus</i>	<i>Salmonella abony</i>	<i>Shigella boydii</i>
Methanol K.M	11.66± 1.15	9.0± 0.00	-	-
Dichloromethane K.M	12.33± 0.57	10.33± 0.57	-	-
Ethyl Acetate K.M	11.33± 0.57	11.33±0.57	-	-
n-hexane K.M	11.33±0.57	11.0±0.00	-	-
Methanol S	11.0 ± 0.00	9.0± 0.00	-	-
Dichloromethane S	11.66 ± 0.57	12.33± 0.57	-	-
Ethyl Acetate S	12.66±0.57	10.0±0.00	-	-
n-hexane S	10.66 ± 0.57	11.0±0.00	-	-
Ciprofloxacin (positive control)	19.0 ± 0.00	22.0±0.00	22.0±0.00	15.0±0.00
Solvent (negative control)	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00

“-” = No zone of inhibition observed; K.M= Kinetic Maceration, S= Soxhlet

The gram- positive bacteria *S. aureus* and *B. cereus* exhibited sensitivity towards the extracts of pericarps as well as seeds obtained by kinetic maceration and soxhlet extraction techniques whereas the gram- negative bacteria *S. abony* and *S. boydii* were found to be resistant. According to Pundir et al., zone of inhibition <9 mm zone was considered as inactive; 9-12 mm as partially active; 13-18 mm as active and >18 mm as very active [17]. From table 1 and 2, it can be observed that most of the extracts fall in the partially active bracket. Amongst all the solvent extracts for *Z. rhetsa* pericarp, ethyl acetate

soxhlet was the most potent (12.66 mm) and this was against *S. aureus*. This was followed by dichloromethane soxhlet and macerated (12.33 mm) against *B. cereus* and *S. aureus* respectively. The ethyl acetate soxhlet and n-hexane soxhlet extracts of seed of *Z. rhetsa*, exhibited the largest zone of inhibition against *B. cereus*. They were found to be more potent as their zone of inhibition was 13mm, indicating that the extracts were active. The antibiotic ciprofloxacin was kept as a positive control and the antibacterial activity of the positive control was also recorded.

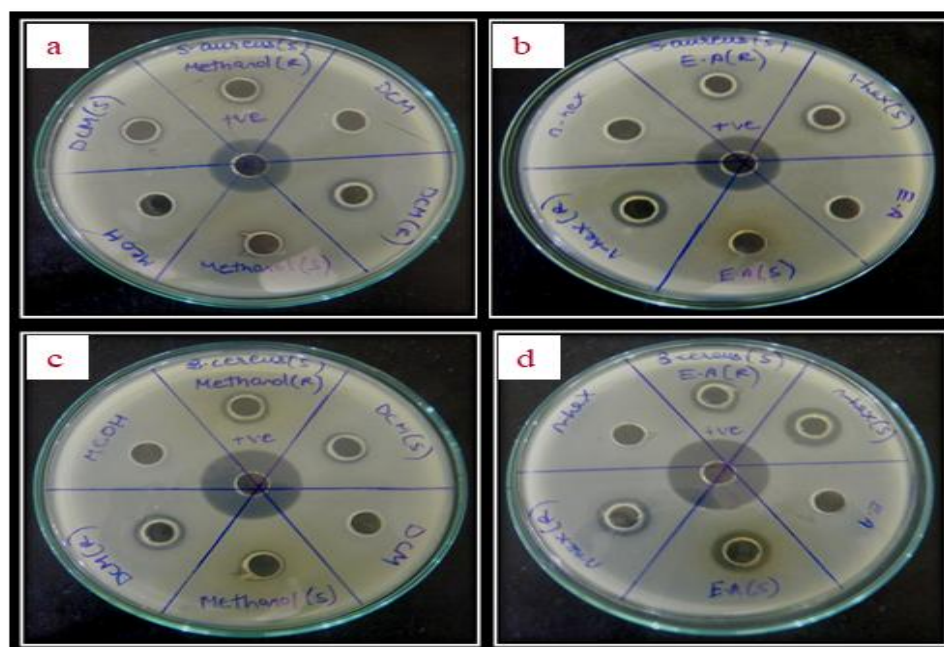


Fig. 2 (a, b): Antimicrobial Activity of *Z. rhetsa* Seed (S) extracts against *S. aureus*
(c, d): Antimicrobial Activity of *Z. rhetsa* Seed (S) extracts against *B. cereus*

Table 2: Zone of inhibition of *Zanthoxylum rhetsa* seed extracts (mm)

<i>Zanthoxylum rhetsa</i> seed	<i>Staphylococcus aureus</i>	<i>Bacillus cereus</i>	<i>Salmonella abony</i>	<i>Shigella boydii</i>
Methanol K.M	9.33 $\pm$ 1.15	10.0 $\pm$ 0.00	-	-
Dichloromethane K.M	11.66 $\pm$ 0.57	11.33 $\pm$ 1.15	-	-
Ethyl Acetate K.M	11.0 $\pm$ 0.00	10.66 $\pm$ 0.57	-	-
n-hexane K.M	11.66 $\pm$ 0.57	11.66 $\pm$ 0.57	-	-
Methanol S	10.0 $\pm$ 0.00	10.0 $\pm$ 0.00	-	-
Dichloromethane S	10.00 $\pm$ 0.57	10.0 $\pm$ 0.00	-	-
Ethyl Acetate S	9.00 $\pm$ 0.00	13.0 $\pm$ 0.00	-	-
n-hexane S	11.00 $\pm$ 0.00	13.0 $\pm$ 0.00	-	-
Ciprofloxacin (positive control)	19.0 $\pm$ 0.00	22.0 $\pm$ 0.00	22.0 $\pm$ 0.00	15.0 $\pm$ 0.00
Solvent (negative control)	0 $\pm$ 0.00	0 $\pm$ 0.00	0 $\pm$ 0.00	0 $\pm$ 0.00

"-" = No zone of inhibition observed; K.M= Kinetic Maceration, S= Soxhlet

Thus, all the plant extracts tested in the present study against food microorganisms displayed narrow spectrum antibacterial activity that is only against the gram-positive bacteria and not gram-negative bacteria. Hence, MIC and MBC values were determined only against the gram-positive bacteria. The difference in the sensitivity between Gram-negative and Gram-positive bacteria may be due to the variation in their cell wall structure. The gram-positive bacteria are found to be more sensitive to herbal extracts as compared to gram-negative bacteria. The inherent tolerance of gram negatives and the nature

and composition of herbs chosen can be the contributing factors. The relative resistance of the gram-negative bacteria can be attributed to the presence of lipopolysaccharide layer and periplasmic space [18].

3.2. Determination of MIC and MBC

The quantification of antibacterial activity was done by determining the MIC and MBC. From the MIC values enlisted in table 3 and 4, it can be observed that all the plant extracts showed antimicrobial activities against *S. aureus* and *B. cereus* with MIC values ranging between 0.312 mg/ml to 12.5 mg/ml. The given sample is said to

be bactericidal in nature when MBC: MIC ≤ 4 and bacteriostatic when this ratio is > 4 [19]. The pericarp extracts were found to be bactericidal as the MBC: MIC was either 4 or less than 4 against both the pathogens. In the case of seed extracts, n-hexane soxhlet showed MBC: MIC of 8 against *S. aureus*, indicating its bacteriostatic nature. Against the pathogen *B. cereus*, all the seed

extracts were found to be bactericidal whereas for *S. aureus* only dichloromethane and ethyl acetate macerated extracts were bactericidal. The MBC values of the remaining extracts viz. methanol, n-hexane macerated extracts as well as methanol, dichloromethane and ethyl acetate soxhlet extracts were observed to be out of the concentration range.

Table 3: MIC, MBC and MBC: MIC ratio of *Zanthoxylum rhetsa* pericarp extracts

<i>Zanthoxylum rhetsa</i> pericarp	<i>Staphylococcus aureus</i>			<i>Bacillus cereus</i>		
	MIC (mg/ml)	MBC (mg/ml)	MBC:MIC	MIC (mg/ml)	MBC (mg/ml)	MBC:MIC
Methanol KM	6.25	6.25	1	6.25	6.25	1
Dichloromethane KM	6.25	12.5	2	6.25	6.25	1
Ethyl Acetate KM	3.12	3.12	1	3.12	3.12	1
n-hexane KM	3.12	3.12	1	3.12	3.12	1
Methanol S	6.25	12.5	2	6.25	6.25	1
Dichloromethane S	3.12	3.12	1	3.12	6.25	2
Ethyl Acetate S	3.12	6.25	2	3.12	6.25	2
n-hexane S	6.25	25.0	4	3.12	3.12	1

Table 4: MIC, MBC and MBC: MIC ratio of *Zanthoxylum rhetsa* seed extracts

<i>Zanthoxylum rhetsa</i> seed	<i>Staphylococcus aureus</i>			<i>Bacillus cereus</i>		
	MIC (mg/ml)	MBC (mg/ml)	MBC:MIC	MIC (mg/ml)	MBC (mg/ml)	MBC:MIC
Methanol KM	6.25	> 25.0	-	12.5	25.0	4
Dichloromethane KM	6.25	25.0	4	3.12	12.5	4
Ethyl Acetate KM	6.25	25.0	4	6.25	6.25	1
n-hexane KM	6.25	> 25.0	-	6.25	6.25	1
Methanol S	12.5	> 25.0	-	6.25	12.5	2
Dichloromethane S	6.25	> 25.0	-	12.5	25.0	2
Ethyl Acetate S	12.5	> 25.0	-	12.5	12.5	1
n-hexane S	3.12	25.0	8	6.25	6.25	1

4. CONCLUSION

The antimicrobial activity against the food pathogens reveals that the plant has potential antimicrobial components and could be studied as a future alternative to control food contamination. For this, it is necessary to screen more pathogens and carry out clinical confirmation and pharmacological standardization. This study will serve as the basis to carry out bioautographic studies to isolate the compounds conferring antibacterial activity, followed by their identification.

5. ACKNOWLEDGEMENTS

The authors are grateful to Guru Nanak Institute of Research and Development, G. N. Khalsa College for providing the facilities to carry out this microbiological work.

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