Phytochemical Investigation and Antibacterial Activity of a Medicinal Plant

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ABSTRACT

Background: *Zanthoxylum rhetsa* (Rutaceae) has many biological activities and is widespread in Bangladesh, China, Indonesia, Malaysia, and India. **Methods:** The total methanolic extract and different successive extracts with phytoconstituents-rich fractions were prepared from *Zanthoxylum rhetsa* fruit by the Soxhlet extraction method. Meanwhile, antibacterial activity was also assessed to identify the most active extracts/fraction against *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus pyogenes*. HR-LCMS was also performed for alkaloid-rich fraction and flavonoid-rich fractions. **Results:** The findings of antibacterial activity indicated that alkaloid-rich and flavonoid-rich fractions were active against all the investigated bacterial strains, and all extracts/fractions were most active against *E. coli* with a higher zone of inhibition. **Conclusion:** The phytoconstituents-rich fraction comprised of a number of alkaloids and flavonoids probably identified for the first time by HR-LCMS. Meanwhile, extracts/fractions also were revealed to have significant activity against *E. coli*, *B. subtilis*, *P. aeruginosa*, *S. aureus*, and *S. pyogenes*.

Key Words: *Zanthoxylum rhetsa, HR-LCMS, Antibacterial activity, Rutaceae, Phytoconstituents.*

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INTRODUCTION

Nature has been the source of therapeutic agents for the remedy of a wide spectrum of ailments all over the world. Plants are considered as a source of novel pharmaceutical products and inexpensive raw material for the synthesis of some known drugs, as they have thousands of active secondary metabolites.[1] These plants with numerous protective mechanisms may be one way in decreasing tissue injury in human disease.[2] The application of plants in the treatment of specific human diseases is evidence for man's ingenuity. Till now, medicinal plants have been utilized in all civilizations as a source of medicine for the control of different diseases including malaria, cancer, AIDS, depression, and stomach complications.[3] They are also utilized to repel insects.[4] As per the World Health Organization (WHO), more than 80% of the population is dependent on medicinal plants in developing countries as an integral part of their primary health care.[5] A noteworthy benefit of the natural products to drug discovery is that it is capable of providing complex molecules that would not be available by other routes. Also, it can provide temple leads for future drug design.[6] Medicinal plants are thus significant sources of synthetic and herbal drugs.

Zanthoxylum rhetsa is a 12 m tall tree, which has conical prickles on its trunk. It is widespread across tropical and subtropical areas of China, India, Indonesia, Bangladesh, and Malaysia. Conventionally, this plant has been applied for various therapeutic applications. It has been applied to improve lactation in breastfeeding mothers and to treat breast pain. The paste prepared from prickly thorns of *Z. rhetsa* is utilized by the Kannikar tribes from Tamil Nadu. The Adi tribes of Arunachal Pradesh, India, consume its shoots as a vegetable. Meanwhile, *Z. rhetsa* also has

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various therapeutic uses. It has been utilized to treat cancer, snake bites, heart diseases, asthma, bronchitis, dermatitis, inflammation, cholera, rheumatism, toothache, lumbago, as well as urinary and venereal complications. It also has stimulant, stomachic, antibacterial, antifungal, digestive, and astringent features.

Primary phytochemical analysis of *Z. rhetsa* revealed the presence of terpenes, flavonoids, alkaloids, coumarins, amides, lignoids, and sterols.[7, 8] Its trunk and root bark were revealed to be a rich source of alkaloids belonging to quinolone and isoquinoline types. Lignoids of furofuranic and diarylbutirolactones types were also reported in abundant amounts. The seed oil was indicated to be efficient in the treatment of cholera and considered useful as anti-inflammatory, disinfectant, and antiseptic agent.[9] 20-episimulanoquinoline, 3,5-dimethoxy-4-geranyloxycinnamyl alcohol, rhetsidimerine, 2,11-didemethoxy vepridimerine B, 8-methoxy-N-methylflindersine, xanthyletin, sesamin, zanthorhetsamide, and chelerybulgarine isolated from the root bark,[10] and 6-acetonyldihydrochelerythrin, arnottianamide, kobusin, columbamine, lupeol, and yangambin isolated from stem bark [7, 8] were tested for anticancer activity. The following compounds were isolated from *Z. rhetsa* plant: rhetsinine dihydroavicine,[11] zanthobungeanine, rutaecarpine, skimmianine, canthin-6-one, N-methylflindersine, dictamine, γ-fagarine, evodiamine,[9] chelerythrine, rhetsine, rhetine,[12] and hydroxyevodiamine [13]from bark; dictamine and arborine [14] from fruits; rutaecarpine [15]from seeds. It has been indicated that the ethanolic extract of *Z. rhetsa* spines has compounds such as diisooctyl ester and 1, 2-benzenedicarboxylic acid as the main compounds, followed by n-hexadecanoic acid and oleic acid. Some volatile compounds were also identified by GC-MS such as α-pinene, 3-elemene, 3-pinene, myrcene, carophyllene oxide, sabinene, spathulenol, 4-terpineol, β-phellandrene, and γ-terpinene from fruits, seeds, and leaves of the plant. The fruit of *Z. rhetsa* is reported for various proven activities like mosquito repellent, and larvicidal, antibacterial, antifungal, antiviral, antioxidant, antitumor, and anti-inflammatory activities.[7]

MATERIALS AND METHODS

Collection and authentication of plant material

The fresh fruits of the plant *Z. rhetsa* were collected from the Udupi district of Karnataka during the period of April 2018. The plant was identified and authenticated by Dr. Rajendra D. Shinde at the Department of Botany, St. Xavier's College, Mumbai.

Drying and powdering of fruits

Around 2 kg of fresh fruit was collected, cleaned, shade dried for two weeks, and powdered. This powder was stored in an air-tight container for further analysis.[16]

Preparation of total methanolic extract

50 g of dried fruit powder was subjected to Soxhlet extraction using methanol at 50-60 °C. The extracts were concentrated with a rotary evaporator under decreased pressure. The dried extract was weighed, and the percent yield of the extracts was calculated.[17]

Preparation of successive extracts

50 g of dried fruit powder was subjected to successive Soxhlet extraction utilizing different organic solvents such as petroleum ether (60-80 °C), dichloromethane, ethyl acetate, and methanol, respectively. The extracts were concentrated with a rotary evaporator under decreased pressure. The dried extract was weighed, and the percent yield of the extracts was calculated.[18]

Preparation of phytoconstituents based rich fraction

a. Preparation of alkaloid-rich fraction:

2 g of total methanolic extract was treated with 1% HCl (v/v) and partitioned against diethyl ether in separating funnel. The water acid phase was made alkaline using concentrated ammonia at controlled pH and the resulting solution was partitioned with chloroform. The alkaloid-rich fraction was revealed to be present in the chloroform fraction. The percent yield was determined. The chloroform extract was dried and analyzed with Dragendorff's reagent for the detection of the presence of alkaloids.[19]

b. Preparation of flavonoids-rich fraction:

2 g of total methanolic extract was admixed with water and filtered. After filtration, the clear aqueous layer was partitioned with petroleum ether in a separating funnel. The aqueous fraction was further partitioned with ethyl acetate. The presence of flavonoids in the ethyl acetate layer was verified by TLC. The ethyl acetate layer was dried and the percent yield was calculated.[20]

IN VITRO ANTIBACTERIAL ACTIVITY

Test Microorganisms and growth media

Escherichia coli, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus pyogenes* were selected for the antibacterial activity. The bacterial strains were provided from the Microbiology Lab of Mithibai College, Mumbai. The bacterial strains were incubated for 24 hours at 37°C in nutrient broth media. The stock cultures were deposited at 4 °C in a refrigerator by preparing nutrient slants.[21, 22]

The zone of inhibition

The antibacterial activity of extracts/fractions was analyzed against three gram-positive (*B. subtilis*, *S. aureus*, and *S. pyogenes*) and two gram-negative bacteria (*E. coli* and *P. aeruginosa*) utilizing the disk diffusion technique. The three set dilutions (10, 30, and 50 mg/ml)

of total methanolic extract, pet ether extract, Dichloromethane extract, ethyl acetate extract, methanol extract, alkaloid-rich fraction, and flavonoid-rich fraction were produced by dissolving in DMSO. Sterilized 6-mm filter paper discs were inoculated with 5 µl of each dilution of extracts. Standard antibacterial discs of ciprofloxacin 5 µg were utilized as the positive control for the investigation. For the negative control, the discs were impregnated with DMSO which was utilized for making dilutions. 10 ml of Mueller-Hinton agar was poured in every Petri plates. After solidification of agar, the fresh bacterial strains were swabbed on the agar surface. The plates were kept for stability for 1 hour in sterile conditions. The previously prepared discs of extracts were placed on these Petri plates. Each plate comprised of at least one negative control and one positive control. The plates were incubated at 37°C for 24 hours. Antibacterial activity was determined in mm as clear inhibitory zone generated around the disc.[21, 22]

HIGH-RESOLUTION LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY (HR-LCMS) ANALYSIS

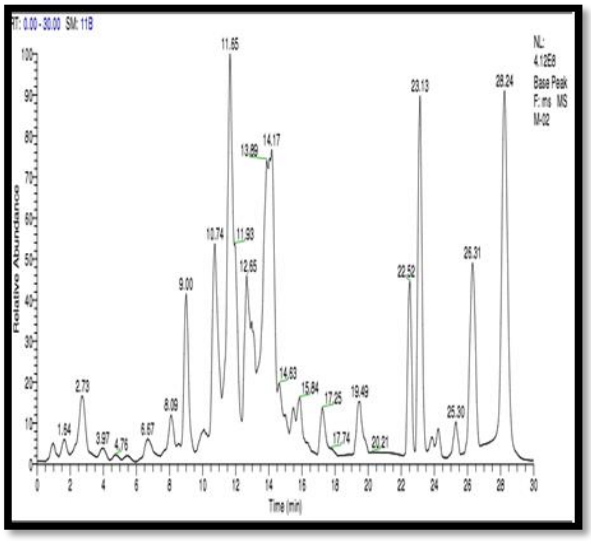
The HR-LCMS of alkaloid-rich and flavonoid-rich fractions was carried out by preparing the fractioned sample in methanol at outsourcing facility in the Sophisticated Analytical Instrument Facility (SAIF), IIT-Bombay, Powai (Mumbai). Chemical fingerprints of two chosen medicinal plant fractions were prepared by Agilent high-resolution liquid chromatography and mass spectrometry (model G6550A) with 0.01% mass resolution. The acquisition method was set to be MS (minimum range of 120 (M/Z) and a maximum range of 1500 Dalton (M/Z) with a scanning rate of each spectrum per second). Gas chromatography was performed at 250 °C with a gas flow of 13 psi/minute.

Hip sampler (model G4226A) was utilized with an auxiliary speed of 100 µl/minute, ejection speed of 100 µl/minute, and flush out factor of 5µl and 3µl injection volume. Within 30 minutes of acquisition time, in the initial 1 minute, the flow of solvent composition A: B was 95:5.

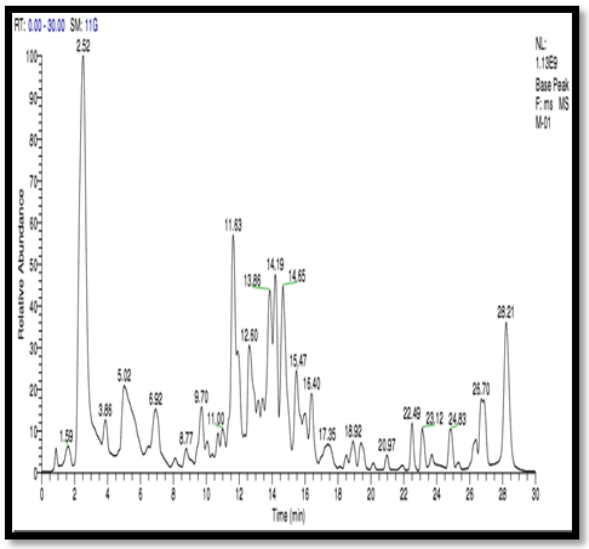
The solvent was used for HR-LCMS.

1. 100% Water
2. 100% Acetonitrile

RESULT AND DISCUSSION



Alkaloid-rich fraction



Flavonoid-rich fraction

Fig 1. Chromatogram of alkaloid-rich fraction and flavonoid-rich fraction

Table 1: Alkaloids identified from the alkaloid-rich fraction

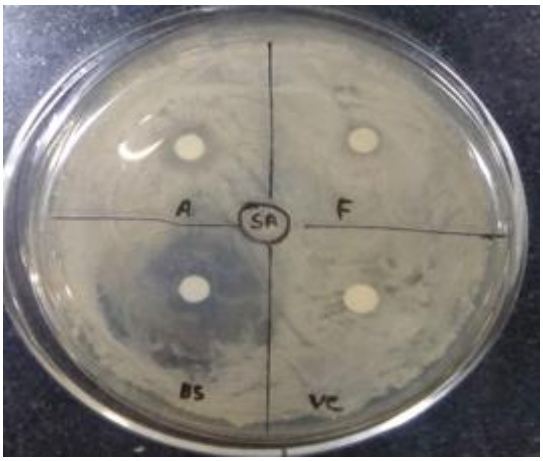
Name of Compound	RT (min)	Mass	Formula
Betaine	0.84	117.07912	$C_5H_{11}NO_2$
Hordenine	0.934	165.11514	$C_{10}H_{15}NO$
Phenethylamine	2.5	121.0892	$C_8H_{11}N$
Ecgonine	4.754	185.10501	$C_9H_{15}NO_3$
Arecoline	7.65	155.0945	$C_8H_{13}NO_2$
Sinomenine	9.00	329.16213	$C_{19}H_{23}NO_4$

Table 2: Flavonoids identified from the flavonoid-rich fraction

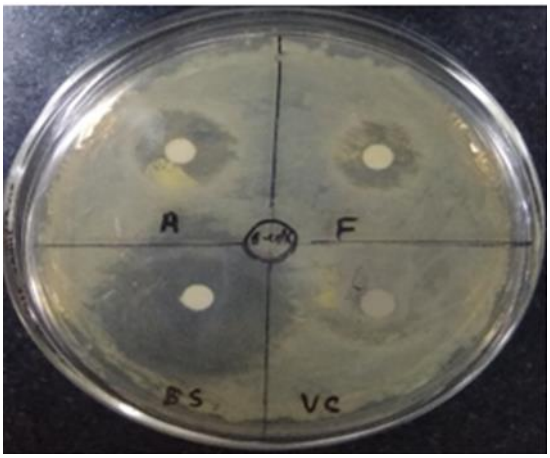
Name of Compound	RT (min)	Mass	Formula
Quercetin	11.535	302.0423	$C_{15}H_{10}O_7$
Hesperidin	12.224	610.189	$C_{28}H_{34}O_{15}$

Table 3: Antibacterial activity of *Zanthoxylum rhetsa*

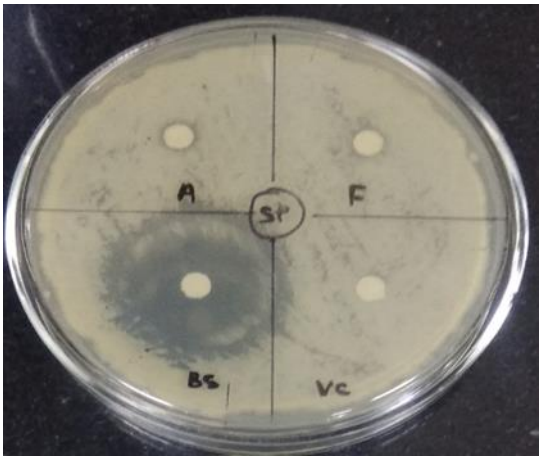
Extract/Fraction	Conc. (mg/ml)	Zone of inhibition (mm)				
		E-coli	*S. aureus*	*Bacillus subtilis*	*P. aurigenosa*	*S. pyogenes*
TME	10	9	0	0	0	0
	30	8	0	0	0	0
	50	8	0	0	0	0
SPEE	10	4	4	0	0	0
	30	6	4	0	0	0
	50	5	6	0	0	0
SDCME	10	7	0	6	0	5
	30	8	0	6	0	4
	50	9	0	8	0	6
SEAE	10	7	5	0	0	5
	30	8	5	0	0	6
	50	9	6	0	0	6
SME	10	4	0	0	0	0
	30	5	0	0	0	0
	50	5	0	0	0	0
Alkaloids rich fraction	10	10	5	0	4	4
	30	11	6	0	5	6
	50	11	5	0	5	4
Flavonoids rich fraction	10	9	4	6	5	4
	30	10	5	5	6	6
	50	9	5	6	6	5
Ciprofloxacin	5 µg	16	18	18	17	18
Vehicle control	-	0	0	0	0	0



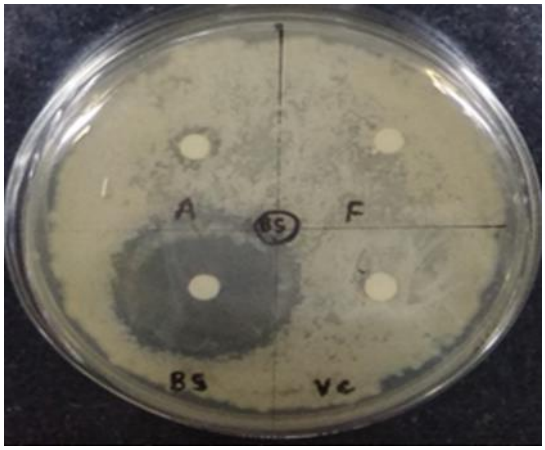
Staphylococcus aureus



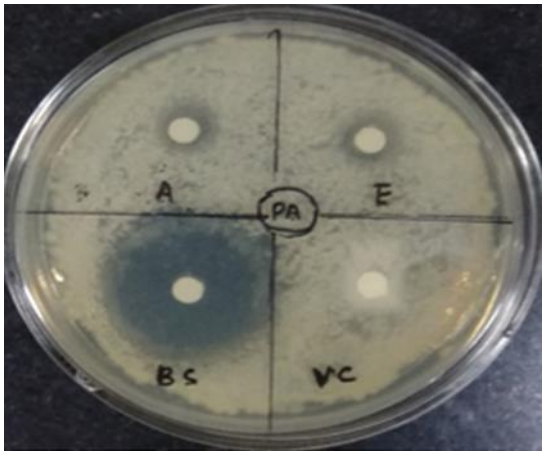
E-coli



Streptococcus pyogenes



Bacillus subtilis



Pseudomonas aeruginosa

Fig 2. Plates tested for antibacterial activity of potent fractions

HR-LCMS analysis of alkaloid-rich and alkaloid-rich fractions of *Z. rhetsa* fruit demonstrated the presence of many phytochemical constituents. According to the data obtained from High-Resolution Liquid Chromatography and Mass Spectra, all these compounds were characterized and probably identified by comparing with reference compounds. Some probable compounds identified as alkaloids were Betaine, Hordenine, Phenethylamine, Ecgonine, Arecoline, and Sinomenine, which were identified from the alkaloid-rich fraction of *Z. rhetsa* fruit. Whereas, only two flavonoids (Quercetin and Hesperidin) were identified from the flavonoid-rich fraction. These compounds are reported for the first time from *Z. rhetsa* fruit.

Antibacterial activity was determined by the disk diffusion method. Based on the obtained results alkaloid-rich and flavonoid-rich fractions were revealed to be active against all the tested bacteria (*E. coli*, *B. subtilis*, *P. aeruginosa*, *S. aureus*, and *S. pyogenes*). Out of all these, alkaloid-rich and flavonoid-rich fractions were revealed to be the most potent against *E. coli* with the highest zone of inhibition compared to other tested samples. The findings obviously reveal that the extract/fraction plays an important role in the treatment of different diseases without any major side effects. So, there is a need to search for new chemical entities or drugs from natural sources. The world is a home of various traditional medicines, from which new drug materials can be obtained. Therefore, to serve new therapeutic agents we need to look back towards those traditional medicines.

CONCLUSION

Based on the HR-LCMS findings, alkaloid-rich fraction and flavonoid-rich fraction contain a number of alkaloids and flavonoids. Extract/fraction of *Z. rhetsa* has proven to demonstrate antibacterial activity against *E. coli*, *B. subtilis*, *P. aeruginosa*, *S. aureus*, and *S. pyogenes.* It is also revealed that some extracts/fractions were much effective towards *E. coli*.

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Conflict of Interest: The authors declare no conflict of interest.

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