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**PHYTOCHEMICAL SCREENING, HPTLC AND BIOAUTOGRAPHY OF  
*ZANTHOXYLUM RHETSA* (ROXB.) DC. EXTRACTS**

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**ABSTRACT**

*Zanthoxylum rhetsa* is an important traditional plant used for various conditions of ailments. The aim of the study was to investigate the physicochemical parameters and antimicrobial potential of the plant. The parameters evaluated were ash values, extractive values, moisture content, phytochemical screening and HPTLC studies. Preliminary phytochemical screening revealed the presence of a variety of phytochemicals. The HPTLC fingerprint pattern was established for ethyl acetate extracts using toluene: ethyl acetate (8:2) (v/v) as solvent and anisaldehyde sulphuric acid as derivatizing reagent. The bioautography results showed that *Z. rhetsa* was rich in antimicrobial compounds. The present work provides invaluable source of information for identification of this plant.

**Keywords: *Zanthoxylum rhetsa*, physicochemical, phytochemical, HPTLC, bioautography, antimicrobial**

**INTRODUCTION**

*Zanthoxylum rhetsa* (Roxb.) DC is a deciduous, medium-sized aromatic tree having conical prickles on the bark of the trunk and branches [1]. The fruits of *Z. rhetsa* are edible and used in traditional medicine for their anti-inflammatory, analgesic, anticonvulsant, and antitumor properties. The seeds promote digestion

and are used in the treatment of stomach ache, toothache, abdominal pain, ascariasis, diarrhoea and dysentery. It also acts as a topical antibacterial agent for treatment of infected wounds [2].

In an effort to discover new lead compounds, plant extracts are screened to detect secondary metabolites with relevant

biological activities [3]. In this regard, bioautography is one of the most effective and inexpensive screening techniques of plant extract analysis. It is the hybridization between planar chromatographic analysis with biological detection method and simple and rapid method for chemical and biological screening [4]. Bioautography is highly efficacious for the detection of antimicrobial compounds because it allows localization of activity even in a complex matrix, facilitating the target-directed isolation of active constituents [5].

*Zanthoxylum rhetsa* has long been valued for its medicinal uses, however, to our best knowledge, there is lack of literature on the antimicrobial activity against food pathogens. In our previous study, the pericarp and seed extracts were screened against four food-associated bacteria [6]. This is the first study using bioautography for identification of antimicrobial compounds in fruit and seed of this plant. Also, this work has been intended to explore the phytochemical screening of solvent extracts and establish fingerprint profile using HPTLC.

## MATERIAL AND METHODS

### Collection and extraction

The fruits of *Z. rhetsa* were collected from APMC market. They were identified by Agharkar Research Institute, Pune, India. The fruits were properly washed, cleaned, shade-dried and kept in the oven. The seeds

were separated and both, seeds and whole fruit were used for the analysis. After complete drying, they were ground to fine powder. Different extracts of the fruit were prepared with methanol, ethanol, water, ethyl acetate, chloroform, petroleum ether.

### Physicochemical evaluation

Percentage of total ash, acid-insoluble ash, water-soluble ash, and loss on drying were calculated. The various extracts prepared were studied for their extractive values [7, 8].

### Preliminary phytochemical analysis

The obtained extracts were subjected to phytochemical screening for the presence or absence of different phytoconstituents viz. triterpenoids, steroids, alkaloids, sugars, tannins, glycosides and flavanoids, etc. by usual prescribed methods [9].

### HPTLC Fingerprinting

The ethyl acetate fruit and seed extract was dissolved in HPLC grade methanol and used for sample application. Chromatographic separation was performed on pre-coated silica gel 60F<sub>254</sub> aluminium sheet. 10 µl of the sample was applied on the plate by CAMAG Linomat V applicator under the pressure of nitrogen gas. The plate was developed in a CAMAG twin trough chamber previously saturated with the mobile phase Toluene: ethyl acetate having ratio (8:2) (v/v) for 15 min. The plate was developed to a distance of 80 mm at room temperature after which it was air-

dried and scanned at wavelength of 254 nm and 366 nm by CAMAG TLC scanner 4 equipped with vision CATS (version 2.5.18262.1) software. Derivatization was achieved by dipping the plate in anisaldehyde sulphuric acid reagent and heating at 105 °C till various colored bands were developed, followed by scanning at 540 nm.

#### **Determination of antibacterial activity by bioautography**

For bioautography, the TLC plate was developed and dried completely to remove traces of solvent. The plate was then overlaid by agar seeded with an overnight culture of *Staphylococcus aureus* ATCC 25923. After solidification, the TLC plate was incubated at 37 °C for 24 hrs. An aqueous solution of tetrazolium salt, p-Iodonitrotetrazolium Violet (INT) was sprayed on the TLC plate and observed for zone of inhibition.  $R_f$  value was calculated and recorded [10, 11].

### **RESULTS AND DISCUSSION**

#### **Physicochemical analysis**

Physicochemical parameters as shown in **Table 1, 2** are useful in determination of adulterants and improper handling of drugs. Ash values, an important criterion and quantitative standard, analyze the identity and purity of crude drugs especially in the powder form [12]. The moisture content is related to stability and shelf life of drug [13]. The extractive values were

determined using solvents of various polarities: methanol, ethanol, water, ethyl acetate, chloroform and petroleum ether (**Table 2**). For both fruit and seed, the water extract had the highest extractive value of 13.58 % and 3.78 % respectively whereas the least extractive value was obtained in the petroleum ether extract that is 2.65 % for fruit and 0.21 % for seed.

#### **Phytochemical screening**

The phytochemical analysis of six different extracts of fruit and seed (**Table 3, 4**) reveals the presence of many medicinally important phytoconstituents such as alkaloids, glycosides, carbohydrates, amino acids, terpenoids and saponins. Flavonoids and reducing sugars are absent in all seed extracts but present in some fruit extracts. The fruit and seed extract lack phenols, tannins and proteins. The presence of phytochemicals indicates that the plant has therapeutic value and can be used to treat various kinds of diseases.

#### **HPTLC Fingerprinting**

Chromatographic fingerprint helps in authentication and correct identification of herbal medicines. Thus, it is very important to obtain reliable chromatographic fingerprints that represent pharmacologically active and chemically characteristic component of the herbal drug. HPTLC fingerprint of ethyl acetate extract of fruit and seed was developed in the mobile phase toluene: ethyl acetate (8:2) (v/v) and

the derivatizing reagent was anisaldehyde sulphuric acid (Figure 1). As seen in Figure 2, chromatogram of fruit extract exhibited 7 peaks with  $R_f$  ranging from 0.047 to 0.844 with a constituent at  $R_f$  0.844 at highest concentration (32.23%). The seed extract chromatogram also exhibited 7 peaks with  $R_f$  ranging from 0.086 to 0.841 and the constituent at  $R_f$  0.403 was present at highest concentration of 44.63 % (Figure 3).

#### Determination of antibacterial activity by bioautography

The bioautography of the separated components in HPTLC were studied for the presence of antibacterial activity and the bioautograms are shown in Figure 4. The

colorless aqueous solution of INT dye reacts with the dehydrogenases enzymes present in the living bacterial cells and converts to intensely colored formazan product [14]. Thus after incubation with *S. aureus* and staining with INT, it can be seen that the separated extracts of *Z. rhetsa* fruit and seed showed the presence of antimicrobial compounds. Areas containing metabolically active bacteria were observed as purple-pink whereas the inhibition of bacterial growth was detected as white bands. Overall, 5 inhibition bands were located at different  $R_f$  values on the bioautogram of *Z. rhetsa* fruit, whereas 3 were seen in the case of *Z. rhetsa* seed.

Table 1: Ash values and loss on drying of powdered fruit and seed of *Zanthoxylum rhetsa*

Sr. No.	Parameters	% w/w (Mean $\pm$ SEM)	
		Fruit	Seed
1.	Total ash	5.00 $\pm$ 0.14	5.15 $\pm$ 0.11
2.	Acid insoluble ash	0.18 $\pm$ 0.02	1.22 $\pm$ 0.12
3.	Water soluble ash	1.48 $\pm$ 0.29	2.94 $\pm$ 0.09
4.	Loss on drying	12.60 $\pm$ 0.19	10.10 $\pm$ 0.36

Table 2: Extractive values with different solvents

Sr. No.	Solvent	% extractability (Mean $\pm$ SEM)	
		Fruit	Seed
1.	Methanol	11.28 $\pm$ 0.27	2.59 $\pm$ 0.24
2.	Ethanol	7.64 $\pm$ 0.69	0.56 $\pm$ 0.08
3.	Water	13.58 $\pm$ 0.30	3.78 $\pm$ 0.39
4.	Ethyl acetate	4.22 $\pm$ 0.08	2.52 $\pm$ 0.15
5.	Chloroform	3.78 $\pm$ 0.22	0.51 $\pm$ 0.04
6.	Petroleum ether	2.65 $\pm$ 0.05	0.21 $\pm$ 0.04

Table 3: Qualitative phytochemical analysis of various extracts of *Z. rhetsa* fruit

Name of phytochemical	Methanol	Ethanol	Water	Ethyl acetate	Chloroform	Petroleum ether
Alkaloids	+	+	-	-	-	-
Flavonoids	+	+	-	+	-	-
Phenols	-	-	-	-	-	-
Tannins	-	-	-	-	-	-
Glycosides	+	+	+	+	+	+
Carbohydrates	+	+	+	+	+	+
Reducing sugars	+	-	+	-	+	-
Proteins	-	-	-	-	-	-
Amino acid	+	+	+	-	-	-
Terpenoids	+	+	+	+	+	-
Saponins	-	-	+	-	-	+

Key: “+”= present; “-” = absent

Table 4: Qualitative phytochemical analysis of various extracts of *Z. rhetsa* seed

Name of phytochemical	Methanol	Ethanol	Water	Ethyl acetate	Chloroform	Petroleum ether
Alkaloids	+	+	-	-	-	-
Flavonoids	-	-	-	-	-	-
Phenols	-	-	-	-	-	-
Tannins	-	-	-	-	-	-
Glycosides	-	-	-	-	+	+
Carbohydrates	+	+	+	+	+	+
Reducing sugars	-	-	-	-	-	-
Proteins	-	-	-	-	-	-
Amino acid	+	+	+	-	-	-
Terpenoids	+	+	+	+	+	-
Saponins	-	-	+	-	-	-

Key: "+= present; - = absent"

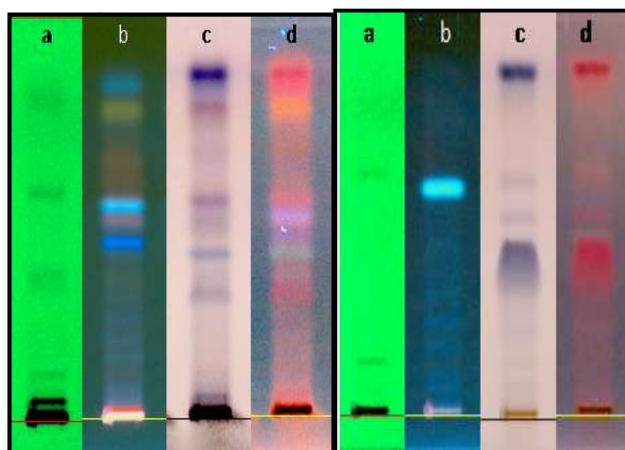


Figure 1: TLC plates of fruit (left) and seed (right) a) under UV 254 nm b) under UV 366 nm c) under visible light 540 nm after derivatization d) under UV 366 nm after derivatization

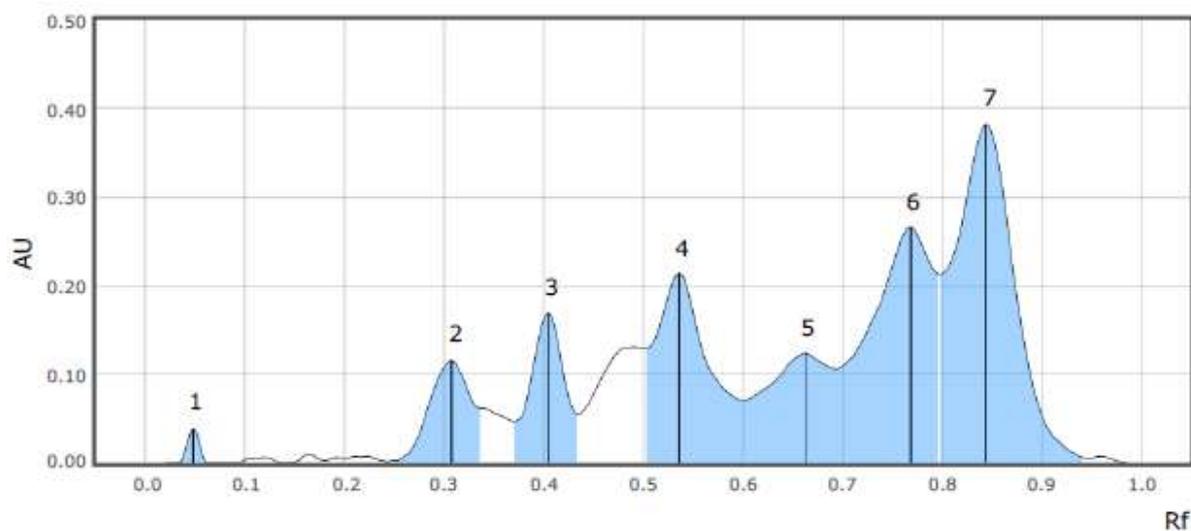


Figure 2: HPTLC chromatogram of ethyl acetate fruit extract after derivatization at 540 nm

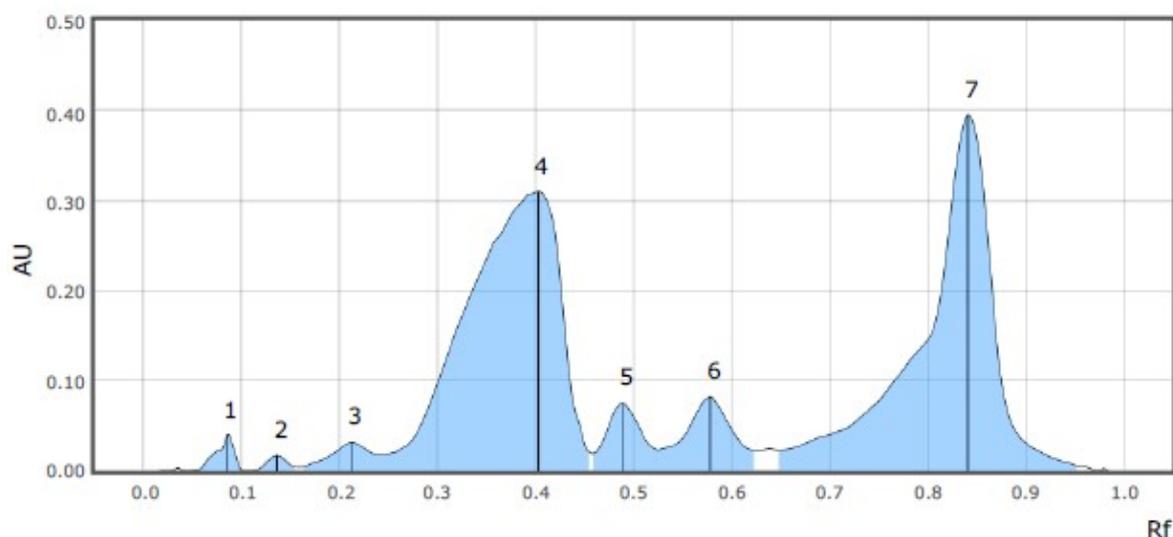


Figure 3: HPTLC chromatogram of ethyl acetate seed extract after derivatization at 540 nm

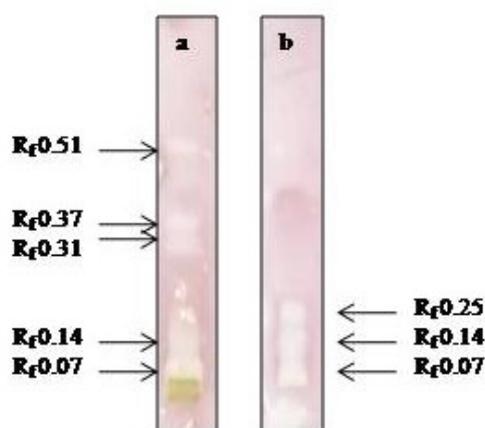


Figure 4: TLC bioautography for antibacterial activity in a) fruit and b) seed

## CONCLUSION

The physicochemical standards like ash values, loss on drying, extractive values are considered to be reliable for crude drugs and were determined in this study. The results evidenced the presence of pharmacologically active phytochemicals such as alkaloids, glycosides, carbohydrates, amino acids, terpenoids and saponins. The chromatographic fingerprint was obtained to standardize the extract of

fruit and seed which would be useful to gauge the quality of crude drugs. The bioautography assay revealed that the fruit and seed of *Z. rhetsa* is a rich and potential source of antimicrobials and hence could be exploited to isolate and identify antimicrobial compounds. The chemical and physicochemical properties represent an indispensable part in authentication as well as the development of herbal drugs from crude material.

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