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## Evaluation of *in vitro* antibacterial property of some plants of subtropical climate against *Rhodococcus equi*

**Lalit Kumar, LN Sankhala, RK Dedar, Lakshmi Kant, DK Badsawal and Sanjay Kumar**

### Abstract

The present study was planned to investigate *in vitro* antibacterial activity of ethanolic, chloroformic and SEWE (Sequentially Extracted Water Extract) extracts of *Capparis decidua* (leafless branches), *Salvadora oleoides* (pod tumours), *Prosopis cineraria* (pod tumours & leaves), *Ailanthus excelsa* (bark & leaves), *Citrullus colocynthis* (L.) Schrad (fruits & leaves) and *Hibiscus rosa-sinensis* L. (leaves) against Vap A and Vap C positive *Rhodococcus equi*. In initial screening ethanolic extract of these plants except *Hibiscus rosa-sinensis* L., were non-active against *R. equi*. Ethanolic and chloroformic leaves extract of *Hibiscus rosa-sinensis* L. showed moderate while SEWE showed good *in vitro* antibacterial activity against *R. equi*. Further, solvent based fractionation of polar and non-polar compounds did not showed *in vitro* antibacterial activity against *R. equi*. On comparison with currently used antibiotics (azithromycin and rifampicin), required concentration of the leaves extract of *Hibiscus rosa-sinensis* L. was too high for their possibilities of *in vivo* use, so abundant availability of *Hibiscus rosa-sinensis* L. leaves and their activity against *R. equi* suggests their potential for use as disinfectant against *R. equi*.

**Keywords:** Chloroform, ethanol, *Hibiscus rosa-sinensis* L., *In vitro*, *Rhodococcus equi*

### 1. Introduction

*Rhodococcus equi* is a gram positive, pleomorphic rod, facultative intracellular pathogen, surviving and replicating in macrophages, commonly found in soil and an important respiratory pathogen of young foals<sup>[11]</sup>. Infection of *R. equi* causes a subacute or chronic abscessating bronchopneumonia<sup>[6]</sup>. The main cause of foal mortalities is *R. equi* infection and on swab sampling from the upper respiratory tract about 17 to 20% foals are PCR positive of *R. equi* infection<sup>[17, 20]</sup>. Erythromycin is replaced by clarithromycin or azithromycin, newer generation macrolides in combination with rifampin<sup>[9]</sup>. There is a concern about the major problems of resistant strains to these drugs<sup>[2, 7, 10, 14, 16, 19]</sup>. The emergence of resistance is stated by increased use of macrolides to control the disease<sup>[22]</sup>. So, it is require to identify novel alternative medicines for treatment of *R. equi* infected foals. One important aspect of alternative medicine includes herbal medicines in which locally available plants or its parts are used for their antimicrobial properties. Plants produce secondary metabolites such as alkaloids, tannins, glycosides, quinines, saponins, flavonoids, terpenoids, steroids and coumarins are the source of plant-derived antimicrobial substances<sup>[5, 25]</sup>. So, the proposed study is planned to (i) initial screening of *in vitro* antibacterial activity of the ethanolic extracts of some selected plant parts of Bikaner region against *R. equi*. and (ii) further polarity and solubility based fractionation of ethanolic extract showing *in vitro* antibacterial activity against *R. equi*.

### 2. Materials and methods

#### 2.1 Initial screening

In the present study, the research work was carried out at ICAR-NRCE (National Research Center on Equine), EPC, Bikaner. In the initial screening, fresh leafless branches of *Capparis decidua* (Kair / Ker), pod tumours of *Salvadora oleoides* (Kharo Jaal / Bada Peelu), pod tumours & leaves of *Prosopis cineraria* (Khejri / Sangria), barks & leaves of *Ailanthus excelsa* (Tree of Heaven / Adusa / Maharukh / Mahaneem / Ghodakaranj), fruits & leaves of *Citrullus colocynthis* (L.) Schrad (Tumba / Bitter Apple / Badi Indrayan), leaves of *Hibiscus rosa-sinensis* L. (Gudhal / Chinese Hibiscus / China Rose / Rose Mallow) were collected

manually from campus of ICAR-NRCE, EPC, Bikaner, dried in hot air oven at 50 °C and grinded in mixer grinder to powder formation. Prepared ethanolic extract <sup>[8]</sup> by using 500 ml absolute ethanol (99.9%) in 50 gram of powder of plant's parts. Than incubated overnight at 37 °C in shaker incubator, sonicated in sonicator and evaporated the filtrate of sonicated extract in the rotary evaporator machine. Weight of the ethanolic extract was measured against absolute ethanol in similar volume.

## 2.2 Polarity based fractionation of the active compound

Further, polarity based fractionation was done to separate non-polar and polar compounds using chloroform and distill water sequential extraction using basic principles <sup>[15]</sup>.

### 2.2.1 Preparation of chloroformic extract for fractionation of non-polar compounds

500 ml chloroform (99.9% pure) was added in 50 gram plant's parts powder and incubated overnight at 37 °C in shaker incubator. Then filtered and residual supernatant was washed with chloroform until clean chloroform was observed and evaporated the filtrate in the rotary evaporator machine. Weight of the chloroformic extract was measured against 99.9% pure chloroform in similar volume.

### 2.2.2 Preparation of Sequentially Extracted Water Extract (SEWE) for fractionation of polar compounds

Chloroformic washed supernatant was spread on the blotting paper for complete drying. 500 ml distilled water was added in dried supernatant, incubated overnight at 37 °C in shaker incubator, sonicated in sonicator and evaporated the filtrate of Sonicated extract in the rotary evaporator machine. Weight of the Sequentially Extracted Water Extract (SEWE) was measured against distilled water in same volume.

## 2.3 Solubility based fractionations

### 2.3.1 Solubility based fractionations of polar compounds of SEWE

Further, solubility based fractionations of polar compounds of SEWE were done with sequentially in ethanol, methanol and distilled water and collected Ethanolic Soluble Fraction (ESF), Methanol Soluble Fraction (MSF) and Water Soluble Fraction (WSF) respectively and tested for their *in vitro* antibacterial activity against *R. equi*.

### 2.3.2 Solubility based fractionations of non-polar compounds of chloroformic extract

Solubility based fractionations of non-polar compounds of chloroformic extract were done with sequentially in petroleum ether, ethyl acetate, chloroform, acetone & ethanol and collected Petroleum Ether Soluble Fraction (PESF), Ethyl Acetate Soluble Fraction (EASF), Chloroform Soluble Fraction (CSF), Acetone Soluble Fraction (ASF) and Ethanol Soluble Fraction (ESF) respectively and tested for their *in vitro* antibacterial activity against *R. equi*.

### 2.4 Evaluation of *in vitro* antibacterial activity

Disc diffusion method <sup>[21, 23]</sup> and agar well diffusion method <sup>[12]</sup> were used to evaluate *in vitro* antibacterial activity of extracts of plant parts against Vap A and Vap C positive *R. equi* using Muller Hinton Broth and Muller Hinton HiVeg Agar. Measured the Inhibition Zone (IZ) diameter to determine the degree of *in vitro* antibacterial activity of plant's parts extract against *R. equi* were as followings:

1. Non Active – when IZ diameter is zero
2. Mild Active – when IZ is < 10 mm diameter
3. Moderate Active – when IZ is > 10 mm and < 15 mm diameter
4. Good Active – when IZ is >15 mm diameter

**2.5 Control:** Azythromycin and rifampicin 10 mg/liter in ethanol were taken as control.

### 2.6 Polymerase Chain Reaction (PCR) Technique

Pure colony of *R. equi* was procured from NCVTC, Hisar and verified time to time for purity by using the PCR technique <sup>[3]</sup>. We obtained the amplified 550 and 700 BP fragments of the *R. equi* pathogenic Vap A and Vap C genes respectively.

## 3. Results and Discussion

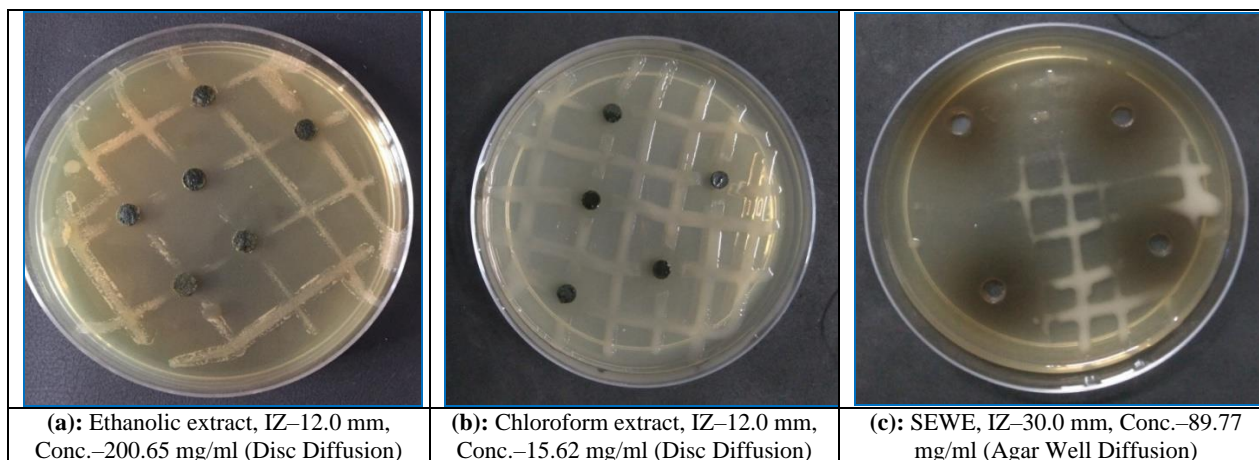
### 3.1 Extract / Fraction of plant's parts

*In vitro* antibacterial activity of ethanolic extract of plant's parts against *R. equi* and further polarity and solubility based fractionation are showing in table 1. In initial screening ethanolic extract of all plants parts except *Hibiscus rosa-sinensis* L., were non-active against *R. equi*. Ethanolic (Fig.1-a) and chloroformic (Fig.1-b) leaves extract of *Hibiscus rosa-sinensis* L. showed moderate while SEWE (Fig.1-c) showed good *in vitro* antibacterial activity against *R. equi*.

**Table 1:** *In vitro* antibacterial activity of plant's parts extract / fraction against *R. equi*

Plant	Part used	Extract / Fraction	Concentration	Method	Inhibition Zone diameter	Degree of <i>in vitro</i> antibacterial activity
<i>Capparis deciduas</i>	Leafless branches	Ethanolic extract	261.5 mg/ml	Disc Diffusion	Zero	None
<i>Salvadora oleoides</i>	Pod tumours	Ethanolic extract	281.5 mg/ml	Disc Diffusion	Zero	None
<i>Prosopis cineraria</i>	Pod tumours	Ethanolic extract	124.5 mg/ml	Disc Diffusion	Zero	None
	Leaves	Ethanolic extract	220.0 mg/ml	Disc Diffusion	Zero	None
<i>Ailanthus excelsa</i>	Bark	Ethanolic extract	129.8 mg/ml	Disc Diffusion	Zero	None
	Leaves	Ethanolic extract	181.5 mg/ml	Disc Diffusion	Zero	None
<i>Citrullus colocynthis</i> (L.) Schrad	Fruits	Ethanolic extract	156.5 mg/ml	Disc Diffusion	Zero	None
	Leaves	Ethanolic extract	135.37 mg/ml	Disc Diffusion	Zero	None
<i>Hibiscus rosa-sinensis</i> L.	Leaves	Ethanolic extract	200.65 mg/ml	Disc Diffusion	12.0 mm	Moderate
		Chloroformic extract	15.62 mg/ml	Disc Diffusion	12.0 mm	Moderate
		SEWE	89.77 mg/ml	Agar Well Diffusion	30.0 mm	Good
		ESF of SEWE	77.25 mg/ml	Agar Well Diffusion	Zero	None
		MSF of SEWE	49.93 mg/ml	Agar Well Diffusion	Zero	None
		WSF of SEWE	142.27 mg/ml	Agar Well Diffusion	Zero	None

		PESF of CE	9.05 mg/ml	Agar Well Diffusion	Zero	None
		EASF of CE	16.89 mg/ml	Agar Well Diffusion	Zero	None
		CSF of CE	36.09 mg/ml	Agar Well Diffusion	Zero	None
		ASF of CE	79.57 mg/ml	Agar Well Diffusion	Zero	None
		ESF of CE	41.0 mg/ml	Agar Well Diffusion	Zero	None



**Fig 1:** *In vitro* antibacterial activity of leaves extract of *Hibiscus rosa-sinensis* L. against *R. equi*: (a) Ethanol extract (b) Chloroformic extract (c) SEWE

### 3.2 Solvents

In the present study, the chemical solvents were used analytical grade. In disc diffusion method, discs were dip in solvents (ethyl alcohol and chloroform) and dry until the solvents were completely evaporate. So the concentration of these chemical solvents in the dry discs were zero.

Ethanol is well known to dissolve both polar and non-polar compounds because of its polar nature due to its hydroxyl group (OH) and non-polar nature due to ethyl (C<sub>2</sub>H<sub>5</sub>) group. Chloroform dissolves non-polar compounds and distilled water dissolves polar compounds.

### 3.3 Non-active plants

In initial screening the ethanolic extract of leaves of leafless branches of *Capparis decidua*, pod tumours of *Salvadora oleoides*, pod tumours & leaves of *Prosopis cineraria*, barks & leaves of *Ailanthus excelsa* and fruits & leaves of *Citrullus colocynthis* (L.) Schrad did not show *in vitro* antibacterial activity against *R. equi*. There are so many factors like environmental factors (pH of the medium, temperature, water activity, oxygen and nutrient availability), choice of solvent, source of the organisms, biochemistry, physiology, metabolism and adaptation strategies of the microbes, plant species, biochemistry, age and parts, concentration of the plant extract and period of extraction, which affect the antimicrobial susceptibility pattern of plant extract [13].

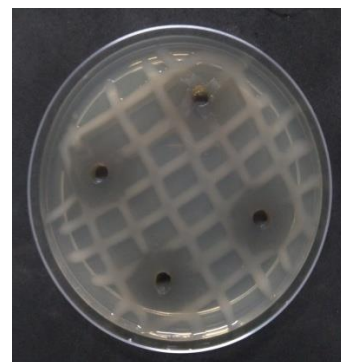
### 3.4 *Hibiscus rosa-sinensis* L.

In initial screening ethanolic extract of leaves of *Hibiscus rosa-sinensis* L. has shown moderate antibacterial activity at 200.65 mg/ml concentration. On sequential fractionation, chloroformic extract (CE) at 15.62 mg/ml concentration and sequentially extracted water extract (SEWE) at 89.77 mg/ml concentration have shown moderate and good antibacterial activity against *R. equi* respectively. Similarly antimicrobial activity of *H. rosa-sinensis* was reported by many workers [18, 24, 26]. In the present findings antibacterial activity of the *H. rosa-sinensis* leaves extract was reported in both polar and non-polar solvents [1]. chloroformic extract (CE) was further fractionated sequentially in petroleum ether, ethyl acetate, chloroform, acetone & ethanol and SEWE was further

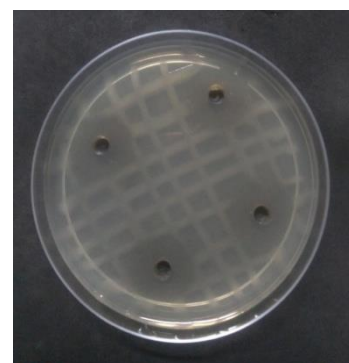
fractionated sequentially in ethanol, methanol & distill water and no fraction showed inhibition zone against *R. equi*, possibly the active components were showing antibacterial activity together in group and on separation they have lost their antibacterial activity.

### 3.5 Control - Azithromycin and Rifampicin

Azithromycin and Rifampicin were taken as control having concentration of 10 mg/L and showed 25.0 mm (Fig.2) and 20.0 mm (Fig.3) diameter of inhibition zone respectively against *R. equi* using agar well diffusion method.



**Fig 2:** Control – Azithromycin IZ – 25.0 mm, conc. - 10.0 mg/L (Agar Well Diffusion Method)



**Fig 3:** Control – Rifampicin IZ – 20.0 mm, Conc. - 10.0 mg/L (Agar Well Diffusion Method)



### 3.6 Comparison with antibiotics

The most effective and prevalent treatment against *R. equi* in foals is combination of macrolides (erythromycin / azythromycin) and rifampicin, but resistant strains of *R. equi* is also being observed [4]. In present experiment, commercially available azythromycin and rifampicin were used at 10 mg/L concentration and both the antibiotics has shown good zone of inhibition. Most effective herbal fraction SEWE of *H. rosa-sinensis* L. leaves has shown good antibacterial activity at 89.77 mg/ml concentration against *R. equi*. It shows that, currently used antibiotics have more antimicrobial efficacy than the most active SEWE fraction of the *H. rosa-sinensis* L. leaves. If the most active SEWE fraction of *H. rosa-sinensis* L. leaves is consider as nontoxic and not interfered by digestive and metabolic processes than it will be use for treatment of foals. So it suggests that there is need to find more purified compound of most active SEWE fraction of *H. rosa-sinensis* L. leaves for to see the possibilities of *invivo* use. However, there are possibilities of direct use of leaves boil water of *H. rosa-sinensis* L. against *R. equi* as farm disinfectant.

### 4. Conclusion

On comparison with currently used antibiotics, required concentration of the most active SEWE fraction of *H. rosa-sinensis* L. leaves is too high for their possibilities for *invivo* use. However, abundant availability of *H. rosa-sinensis* L. leaves and their activity against *R. equi* suggests their potential for use as disinfectant against *R. equi*.

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

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