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Screening for *In Vitro* Antibacterial Activity of Leaves Extracts of Certain Selected Plants against *Rhodococcus equi*

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ABSTRACT

In the present study, the research work was carried out during June 2018 to March 2019 at ICAR-NRCE-EPC (Indian Council of Agricultural Research, National Research Centre on Equines, Equine Production Campus), Jorbeer, Bikaner (Rajasthan), India. The study was conducted to investigate *in vitro* antibacterial activity of ethanolic, chloroformic and Sequentially Extracted Water Extract (SEWE) leaves extracts of *Aegle marmelos*, *Morus alba*, *Nerium indicum*, *Cascabela thevetia* (L.) Lippold, *Plumeria alba* L. and *Azadirachta indica* against Vap A and Vap C positive *Rhodococcus equi.* Fresh leaves of these plants were collected manually from campus of ICAR-NRCE-EPC, Jorbeer, Bikaner (Rajasthan). In initial screening ethanolic leaves extract of these plants except *Azadirachta indica*, were found non-active against *Rhodococcus equi*. Chloroformic leaves extracts of *Azadirachta indica* did not showed in vitro antibacterial activity against *Rhodococcus equi*. While ethanolic and Sequentially Extracted Water Extract (SEWE) leaves extracts of *Azadirachta indica* showed good *in vitro* antibacterial activity against *Rhodococcus equi*. Further, solvent based fractionation, Ethanol Soluble Fraction (ESF), Methanol Soluble Fraction (MSF) and Water Soluble Fraction (WSF) of polar compounds of SEWE did not showed *in vitro* antibacterial activity against *Rhodococcus equi*. On comparison with currently used antibiotics (azithromycin and rifampicin), required concentration of the leaves extract of *Azadirachta indica* was too high for their possibilities of *in vivo* use, so abundant availability of *Azadirachta indica* leaves and their activity against *Rhodococcus equi* suggests their potential for use as disinfectant against *Rhodococcus equi*.

KEYWORDS: Antibacterial, *Azadirachta indica*, *in vitro*, leaves, *Rhodococcus equi*

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1. INTRODUCTION

Rhodococcus equi is a Gram positive, pleomorphic, rod shaped bacteria. It is an important pathogen of young foals and commonly found in soil. *R. equi* infection can lead to chronic and severe pyogranulomatous pneumonia in young horses, subacute or chronic abscessating bronchopneumonia sometimes with ulcerative typhlocolitis and may include mesenteric lymphadenitis, osteomyelitis, purulent arthritis, reactive arthritis and ulcerative lymphangitis (Dedar et al., 2017). *R. equi* is a facultative intracellular pathogen susceptible to neutrophil mediated killing. It is able to surviving and replicating in macrophages, resists innate macrophage defenses and establishes residence within the intracellular environment of that phagocyte (Hondalus, 1997).

R. equi is an important cause of foal mortalities and about 17–20% foals are PCR positive on swab sampling from the upper respiratory tract in the studies carried out by Kishor Kumar and Irfan Ahmad Mir in Rajasthan and Jammu & Kashmir respectively (Kumar et al., 2014, Mir et al., 2015). At present time there are so many antibiotics are discovered. The most important advances of modern science considered the development of antibiotics (Marston et al., 2016). The combination of rifampin and erythromycin used to treat the disease (Sweeney et al., 1987, Hillidge, 1987). Recenly clarithromycin or azithromycin, newer generation macrolides replaces the erythromycin in combination with rifampin (Gigue`re et al., 2004).

Acquired resistance genes may enable a bacterium to produce enzymes that prevent the drug from reaching its intracellular target, to modify the drug's target site, or to produce an alternative metabolic pathway that bypasses the action of the drug. Conjugation, transformation or transduction is method of acquisition of new genetic material by antimicrobial susceptible bacteria from resistant strains of bacteria (Tenover, 2006). The overuse & abuse of antibiotics, lack of information on pathogens and limited surveillance have contributed to the global epidemic of antibiotic resistance (Fletcher, 2015, Cisek et al., 2014).

It is stated that increased used of macrolides to control the disease have contributed to the emergence of resistance (Pauw and Eloff, 2014). Resistant strains to either of these drugs have also been encountered (McNeil and Brown, 1992, Fines et al., 2001, Kotze and Eloff, 2002, Asoh et al., 2003, Jacks, 2003, Gigue`re et al., 2010, Pauw and Eloff, 2014). The lack of effective alternatives against *R. equi* makes it compulsive to identify novel antimicrobial agents to control and treat *R. equi* infection in foals.

The discussion concludes that some of the common issues are often overlooked and whilst there are numerous opportunities for environmental factors to contribute to the growing burden of antimicrobial resistance, a renewed focus on innovative and traditional environmental approaches is needed to tackle the problem.

Herbal therapy (Rasayan Chikitsa) is an ancient process of traditional treatment (Basak et al., 2020). The variety of plant derived compounds provides very diverse chemical structures that may supply both the novel mechanisms of antimicrobial action. The rapid development of modern biotechnologies opens up the way for obtaining bioactive compounds in eco friendly and low toxic conditions (Gorlenko et al, 2020). Plants have many secondary metabolites like phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, tannins, coumarins, glucosinolates, terpenoids, phenylpropanoids, alkaloids, camalexin, saponins, terpenes, glycosides, carbohydrates and steroids (Kliebenstein, 2004, Maneesha et al., 2021, Sharma et. al., 2021). The secondary metabolites are important source of antimicrobial substances and useful in the treatment of bacterial infections (Srivastava et al., 2014, Fernebro, 2011).

Bikaner has a large diversity of plant species. So we planned to screen the *in vitro* antibacterial activity of extracts of some plants in Bikaner region to identify *in vitro* antimicrobial activity against *R. equi*, which could be further exploited for isolation of phytochemicals for treatment of foals or disinfection of stables.

2. MATERIALS AND METHODS

2.1. Initial screening

In the present study, the research work was carried out during June 2018 to March 2019 at ICAR-NRCE-EPC (Indian Council of Agricultural Research, National Research Centre on Equines, Equine Production Campus), Jorbeer, Bikaner (Rajasthan). In the initial screening, fresh leaves of *Aegle marmelos* (Bael), *Morus alba* (White mulberry / Sahtoot), *Nerium indicum* (Red kaner), *Cascabela thevetia* (L.) Lippold (Pilli kaner / yellow oleander / lucky nut / suicide tree), *Plumeria alba* L. (Nagchampa) and *Azadirachta indica* (Neem) were collected manually from campus of ICAR-NRCE, EPC, Jorbeer, Bikaner, dried in hot air oven at 50^0C and grined in mixer grinder to powder formation. Prepared ethanolic extract by using 500 ml absolute ethanol (99.9%) in 50 gram of powder of plant leaves. Then it was incubated overnight at 37^0C 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 (Jeyaseelan et al., 2012).

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

500 ml chloroform (99.9% pure) was added in 50 g plant's parts powder and incubated overnight at 37^{0}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^{0}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 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 Ethanol Soluble Fraction (ESF), Methanol Soluble Fraction (MSF) and Water Soluble Fraction (WSF) respectively and tested for their *in vitro* antibacterial activity against *R. equi*.

2.4. Evaluation of in vitro antibacterial activity

Disc diffusion method (Nostro et al., 2000, Salie et al., 1996) and agar well diffusion method (Irshad et al., 2012) 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:

Non Active- when IZ diameter is zero

Mild Active- when IZ is less than 10 mm diameter

Moderate Active- when IZ is greater than 10 mm and less than 15 mm diameter

Good Active- when IZ is greater than 15 mm diameter

2.5. Control

Azythromicin and rifampicin 10 mg L^{-1} 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. 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

In present study, pure colony of *R. equi* was procured from National Center for Veterinary Type Cultures (NCVTC), National Research Centre on Equines (NRCE), Hisar and verified time to time for purity by using PCR based on pathogenic Vap A and Vap C genes. By the PCR technique, we obtained the amplification of 550 and 700 bp fragments of the *R. equi* pathogenic Vap A and Vap C genes respectively. These pathogenic Vap A and Vap C genes indicated the colony of the *R. equi* was pure.

3.1. Extract / Fraction of plant's parts

In vitro antibacterial activity of ethanolic leaves extract of *Aegle marmelos* (Bael), *Morus alba* (White mulberry / Sahtoot), *Plumeria alba* L. (Nagchampa), *Nerium indicum* (Red kaner), *Cascabela thevetia* (L.) Lippold (Pilli kaner / yellow oleander / lucky nut / suicide tree) and *Azadirachta indica* (Neem) against *R. equi* and further polarity and solubility based fractionation are showing in table 1. In initial screening ethanolic leaves extract of all these plants except *Azadirachta indica* were non-active against *R. equi* (Figure 1). Chloroformic (Figure 2b) leaves extract of *Azadirachta indica* did not showed *in vitro* antibacterial activity against *R. equi* while Ethanolic (Figure 2a) and SEWE (Figure 2c) showed good *in vitro* antibacterial activity against *R. equi*. Further, solubility based fractionations, Ethanol Soluble Fraction (ESF), Methanol Soluble Fraction (MSF) and Water Soluble Fraction (WSF) of polar compounds of SEWE of *Azadirachta indica* leaves were did not showed *in vitro* antibacterial activity against *R. equi* (Figure 3).

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_2H_5) group. Chloroform dissolves non-polar compounds and distilled water dissolves polar compounds.

3.3. Non-active plants

In initial screening the ethanolic leaves extract of *Aegle marmelos* (Bael), *Morus alba* (White mulberry / Sahtoot), *Nerium indicum* (Red kaner), *Cascabela thevetia* (L.) Lippold (Pilli kaner / yellow oleander / lucky nut / suicide tree) and *Plumeria alba* L. (Nagchampa) did not show *in vitro* antibacterial activity against *R. equi* (Figure 1). There are so many factors like environment, pH of the medium,

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

Plant	Part used	Extract / Fraction	Concentration	Method	Inhibition Zone diameter	Degree of *in vitro* antibacterial activity
Aegle marmelos	Leaves	Ethanolic Extract	122.67 mg ml^{-1}	Disc Diffusion	Zero	None
Morus alba	Leaves	Ethanolic Extract	98.59 mg ml^{-1}	Disc Diffusion	Zero	None
Plumeria alba L.	Leaves	Ethanolic Extract	345.65 mg ml^{-1}	Disc Diffusion	Zero	None
Nerium indicum	Leaves	Ethanolic Extract	131.8 mg ml^{-1}	Disc Diffusion	Zero	None
Cascabela thevetia (L.) *Lippold*	Leaves	Ethanolic Extract	416.33 mg ml^{-1}	Disc Diffusion	Zero	None
Azadirachta indica	Leaves	Ethanolic Extract	89.0 mg ml^{-1}	Disc Diffusion	20.0 mm	Good
		Chloroformic Extract	0.625 mg ml^{-1}	Disc Diffusion	Zero	None
		SEWE	137.37 mg ml^{-1}	Agar Well Diffusion	16.0 mm	Good
		ESF of SEWE	29.82 mg ml^{-1}	Agar Well Diffusion	Zero	None
		MSF of SEWE	70.02 mg ml^{-1}	Agar Well Diffusion	Zero	None
		WSF of SEWE	127.17 mg ml^{-1}	Agar Well Diffusion	Zero	None

Leaves of *Aegle marmelos*

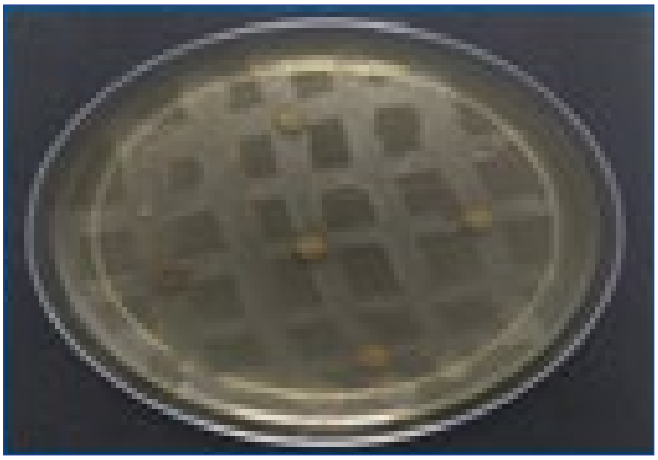
Leaves of *Morus alba*

Leaves of *Plumeria alba* L.

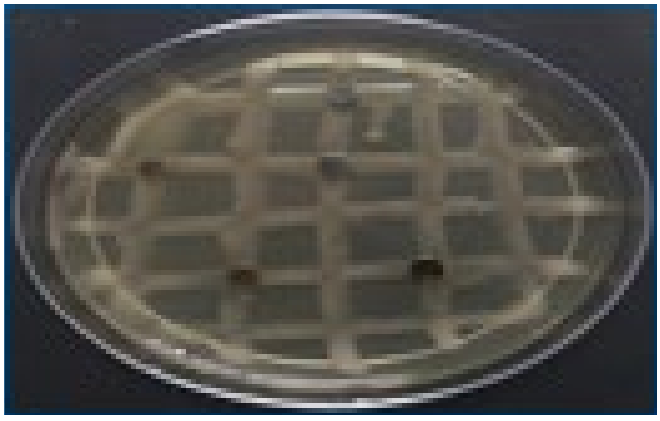
Leaves of *Nerium indicum*

Figure 1: Continue...

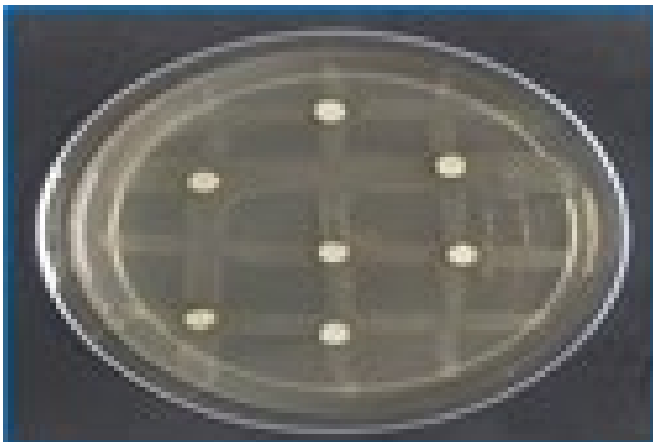
Leaves of *Cascabela thevetia* (L.) Lippold



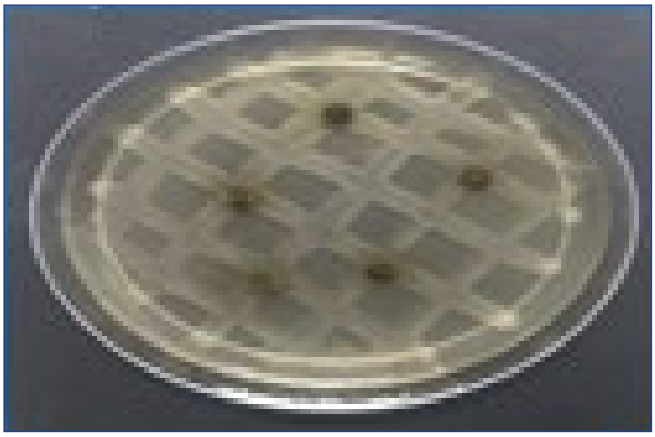
(a) Ethanolic Extract of leaves of *A. marmelos* IZD- 0.0 mm Conc.- 122.67 mg ml^{-1} (Disc Diffusion)



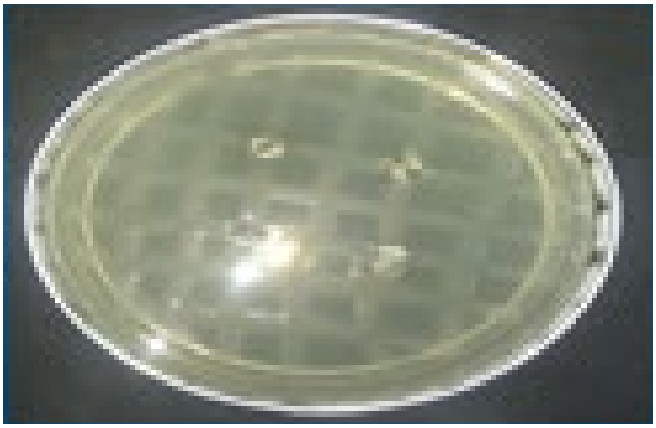
(b) Ethanolic Extract of leaves of *Morus alba* IZD- 0.0 mm Conc.- 98.59 mg ml^{-1} (Disc Diffusion)



(c) Ethanolic Extract of leaves of *P. alba* L. IZD- 0.0 mm Conc.- 345.65 mg ml^{-1} (Disc Diffusion)



(d) Ethanolic Extract of leaves of *N. indicum* IZD- 0.0 mm Conc.- 131.8 mg ml^{-1} (Disc Diffusion)

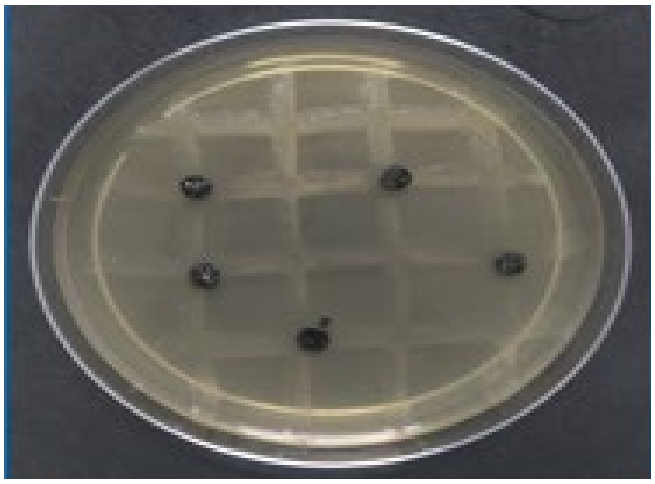


(e) Ethanolic Extract of leaves of *C. thevetia* IZD- 0.0 mm Conc.- 416.33 mg ml^{-1} (Disc Diffusion)

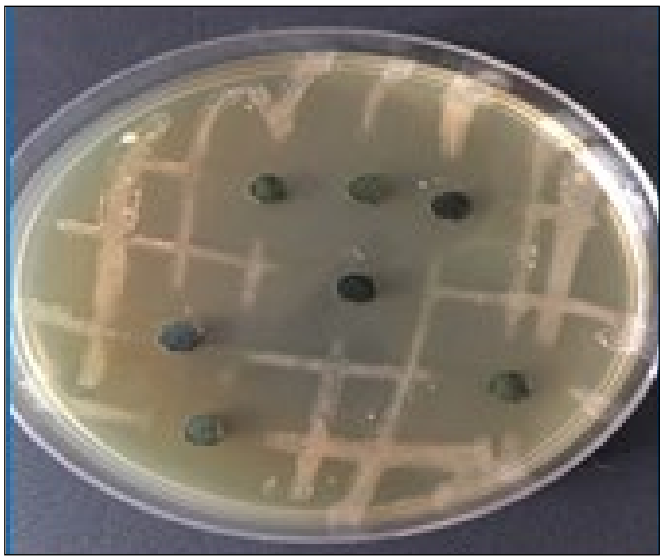
Figure 1: *In vitro* antibacterial activity of Ethanolic Extract of leaves of (a) *Aegle marmelos*; (b) *Morus alba*; (c) *Plumeria alba* L.; (d) *Nerium indicum* and (e) *Cascabela thevetia* (L.) Lippold against *R. equi*

temperature, water activity, oxygen availability, nutrient availability, choice of solvent, source of the organisms, biochemistry, physiology, metabolism, adaptation strategies of the microbes, plant species, age, parts, concentration of the plant extract and period of extraction, which affect the antimicrobial susceptibility pattern of

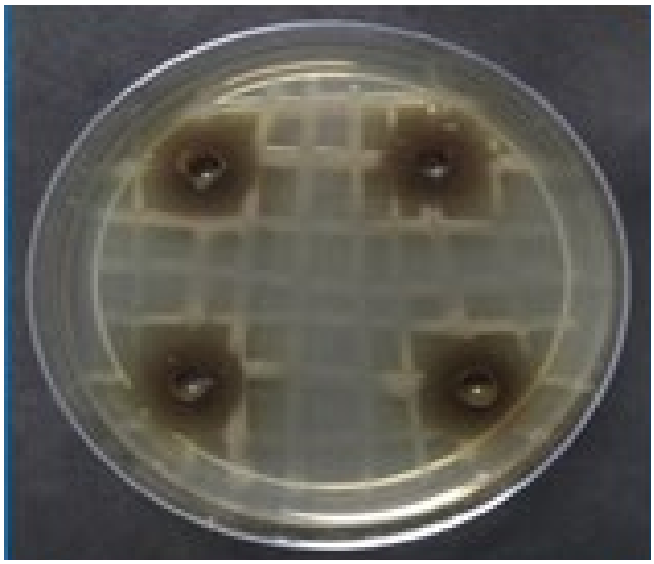
Leaves of *Azadirachta indica*



(b) Chloroformic Extract of leaves IZD- 0.0 mm Conc.- 0.625 mg ml^{-1} (Disc Diffusion)



(a) Ethanolic Extract of leaves IZD- 20.0 mm Conc.- 89.0 mg ml^{-1} (Disc Diffusion)



(c) SEWE of leaves IZD -16.0 mm Conc.- 137.37 mg ml^{-1} (Agar Well Diffusion)

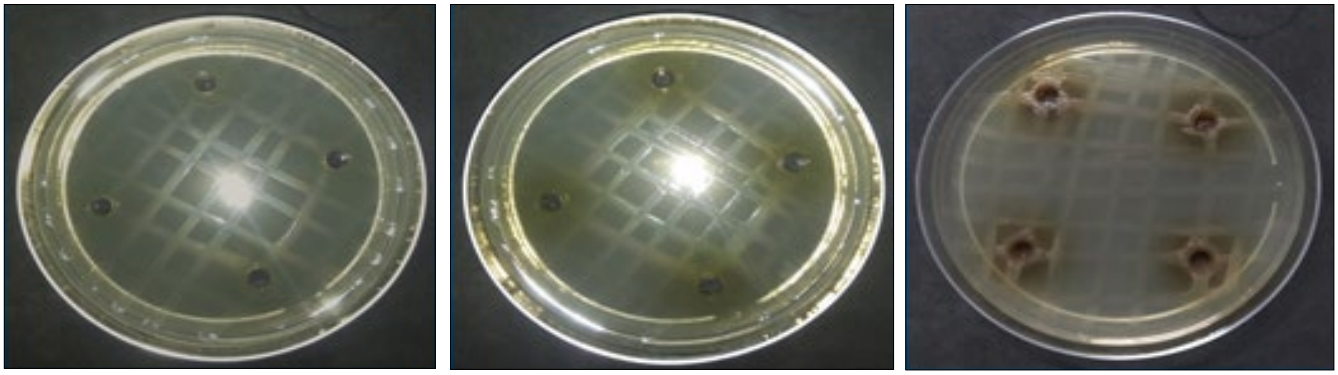
Figure 2: *In vitro* antibacterial activity of leaves extract of *Azadirachta indica* against *R. equi*: (a) Ethanolic Extract; (b) Chloroformic Extract and (c) SEWE

plant extract (Izah, 2018).

3.4. Azadirachta indica (Neem)

In present study, ethanolic leaves extract of *Azadirachta indica* has shown good antibacterial activity against *R. equi* (Figure 2a). Antibacterial activity of alcoholic extract of *A. indica* have been reported against many bacteria and were found comparable to chlorhexidine (Mistry et al., 2014). In present study, on sequential extraction, chloroform extract was found ineffective (Figure 2b) while sequential extracted water extract (SEWE) found good effective against *R. equi* (Figure 2c). It shows that effective components of *A. indica* are polar in nature.

Ethanolic, methanolic, chloroformic and aqueous leaf extract of *A. indica* exhibited antimicrobial activity against different microorganisms (Reddy et al., 2013, Raut et al. 2014, Rajasekaran, 2008, Koona et al., 2011). Phytochemical analysis of Ethanolic, Methanolic, Acetonic and Aqueous leaves extract of *A. indica* gave positive results for lipid, steroids, triterpinoids, reducing sugars, alkaloids, phenolic compounds, flavonoids, tannins, proanthocyanidin, glycosides and coumarin (Susmitha et al., 2013, Vinoth et al., 2012). In the present study, aqueous leaves extract of *A. indica* has been reported for antimicrobial activity against pathogenic microorganism R. *equi*. Further, dilutions of SEWE concentrations up to 70 mg ml^{-1} was found effective against R. *equi*.



(a) ESF of SEWE IZD- 0.0 mm Conc.- 29.82 mg ml^{-1} (Agar Well Diffusion)

(b) MSF of SEWE IZD- 0.0 mm Conc.- 70.02 mg ml^{-1} (Agar Well Diffusion)

(c) WSF of SEWE IZD- 0.0 mm Conc.- 127.17 mg ml^{-1} (Agar Well Diffusion)

Figure 3: *In vitro* antibacterial activity of solvent based fractionation of SEWE of *Azadirachta indica* leaves against *R. equi*: (a) ESF of SEWE; (b) MSF of SEWE and (c) WSF of SEWE

3.5. Control: Azithromycin and Rifampicin

Azithromycin and Rifampicin were taken as control having concentration of 10 mg l^{-1} and showed 25.0 mm (Figure 4) and 20.0 mm (Figure 5) diameter of inhibition zone respectively against *R. equi* using agar well diffusion method.

3.6. Comparison with antibiotics

The combination of Macrolides (erythromycin / azythromicin) and rifampicin is the most effective and prevalent treatment against *R. equi* in foals, but resistant strains of *R. equi* is also being observed (Cisek *et al.*, 2014). In present experiment, commercially available azythromicin and rifamipicin was used @ 10 mg l^{-1} and both the antibiotics

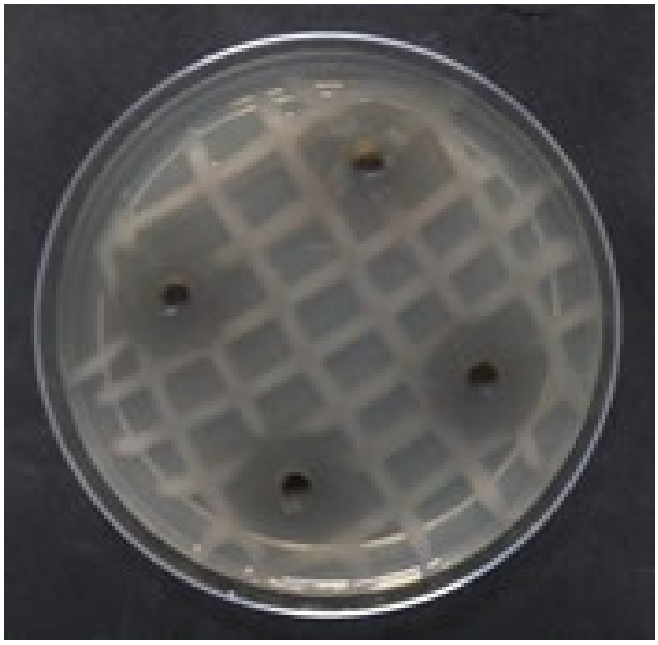


Figure 4: Control Azithromycin IZ- 25.0 mm conc.- 10.0 mg L^{-1} (Agar Well Diffusion Method)

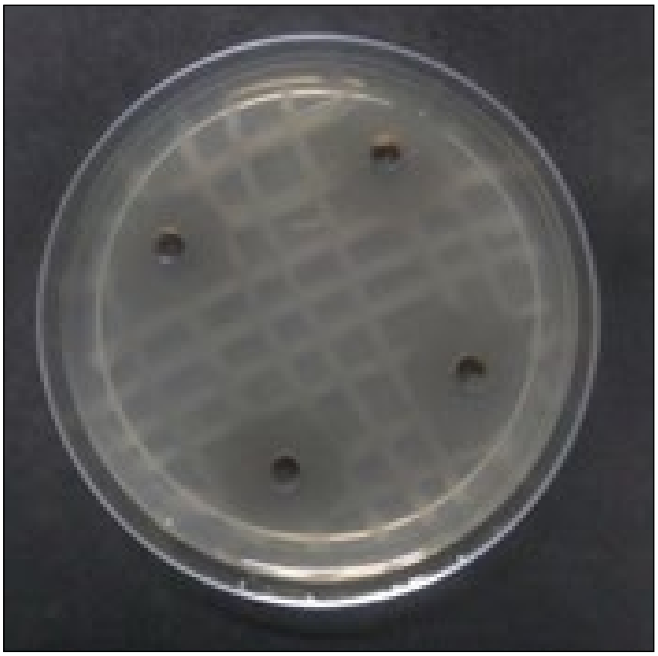


Figure 5: Control Rifampicin IZ- 20.0 mm Conc.- 10.0 mg L^{-1} (Agar Well Diffusion Method)

have shown good zone of inhibition (Figure 4 and Figure 5). While most effective herbal fraction SEWE of *A. indica* leaves showed their minimum inhibitory concentration at 70 mg ml^{-1}. It shows that, quantitatively currently used antibiotics have more times antimicrobial efficacy than the fraction SEWE of *A. indica* leaves. It depicts that even if the extracts are considered nontoxic and not interfered by digestive and metabolic processes than there will be use as antimicrobial agent against *R. equi* in foals. So it suggests that there is need to find more purified compound of these extracts for to see the possibilities of *in vivo* use. However, there are possibilities of direct use of *A. indica* leaves and their water extract against *R. equi* as farm disinfectant.

4. CONCLUSION

On comparison with currently used antibiotics, required concentration of the most active SEWE fraction of *A. indica* leaves is too high for their possibilities for *in vivo* use. However, abundant availability of *A. indica* leaves and their activity against *R. equi* suggests their potential for use as disinfectant against *R. equi*.

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