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Evaluation of *in vitro* antibacterial activity of leaves extracts of *Thespesia populnea, Pongamia pinnata* (L.) Pierre, *Albizia lebbeck* (L.) Benth., *Delonix regia* (Hook.) Raf., *Cordia dichotoma* and *Dalbergia sissoo* against *Rhodococcus equi*

Lalit Kumar, LN Sankhala, Mukani Kumari, RA Legha and RK Dedar

Abstract
The experiment was conducted to evaluation of *in vitro* antibacterial properties of ethanolic, chloroformic and Sequentially Extracted Water Extract (SEWE) leaves extract of *Thespesia populnea, Pongamia pinnata* (L.) Pierre, *Albizia lebbeck* (L.) Benth., *Delonix regia* (Hook.) Raf., *Cordia dichotoma* and *Dalbergia sissoo* against Vap A and Vap C positive *Rhodococcus equi.* In initial screening using disc diffusion method ethanolic leaves extract of *Thespesia populnea, Pongamia pinnata* (L.) Pierre, *Albizia lebbeck* (L.) Benth. and *Delonix regia* (Hook.) Raf. Were found non-active against *R. equi* while ethanolic leaves extract of *Cordia dichotoma* and *Dalbergia sissoo* were showed good *in vitro* antibacterial against *R. equi.* On polarity based fractionation, only chloroformic leaves extract of *Dalbergia sissoo* was showed good *in vitro* antibacterial activity against *R. equi* using disc diffusion method. Further, solubility based fractionations of chloroformic leaves extract of *Dalbergia sissoo* sequentially extracted in petroleum ether, ethyl acetate, chloroform, acetone & ethanol, were found non active against *R. equi* using agar well diffusion method. On comparison with currently used antibiotics (azithromycin and rifampicin), required concentration of the chloroformic leaves extract of *Dalbergia sissoo* was too high for their possibilities of *in vivo* use. However, abundant availability of *Dalbergia sissoo* leaves and their antibacterial activity against *R. equi* suggests their potential for use as disinfectant against *R. equi* and further more investigation will be require phytochemical analysis for isolation of bioactive constituents responsible for their antimicrobial activity.

Keywords: Chloroform, *Cordia dichotoma*, *Dalbergia sissoo*, ethanol, *in vitro* and *Rhodococcus equi*

Introduction
The basic concept of plant products existed in the ancient Vedic scripture the Ayurveda and has been practiced in Indian traditional medicine for many centuries. Ayurveda have two main approaches are preventive and curative [11]. Medicinal plants are the "backbone" of traditional medicine [1]. This new methodology can make it detection, improvement and recognize beneficial effects of herbal preparations [9].

Rhodococcus equi is a Gram-positive, pleomorphic rod, commonly found in soil that is an important pathogen of young foals. *R. equi* infection causes a subacute or chronic abscessating bronchopneumonia, sometimes with ulcerative typhlocolitis, and may include mesenteric lymphadenitis, osteomyelitis, purulent arthritis, reactive arthritis, and ulcerative lymphangitis [10]. *R. equi* is an important cause of foal mortalities and about 17 to 20% foals are PCR positive on swab sampling from the upper respiratory tract in the studies carried out by Dr. Kishor Kumar in Rajasthan [24] and Dr. Irfan Ahmad Mir in Jammu & Kashmir [26].

R. equi is a facultative intracellular pathogen surviving and replicating in macrophages. The combination of rifampin and erythromycin used to treat the disease [17, 34]. Recenly clarithromycin or azithromycin, newer generation macrolides replaces the erythromycin in combination with rifampin [15]. Resistant strains to either of these drugs have also been encountered [2, 13, 16, 20, 23, 25, 30]. It is stated that increased used of macrolides to control the desease have contributed to the emergence of resistance [30]. 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 increasing incidence of microorganisms becoming resistant to antibiotics has continuously become a scientific community concern to identify and isolate new bioactive compounds from medicinal plants using standardized modern analytical procedures.

It could provide novel straightforward approaches against pathogenic bacteria [35]. Many plant secondary compounds are known to have diverse antimicrobial activities against many different pathogens [8]. Plants have many phytochemical constituents such as tannins, saponins, phlobatannins, flavonoids, anthraquinones, terpenoids, steroids, alkaloids, carbohydrates, glycosides, polyphenols (phenolic acids, lignanes, coumarins), terpenes, sapogenins and amines [3, 12, 37]. These phytochemical constituents, which are secondary metabolites and are used for the treatment of many diseases including bacterial infections [5, 27, 36].

So the proposed study was planned to screen the *in vitro* antibacterial activity of extracts of some locally available plants in Bikaner and to identify plants having *in vitro* antimicrobial activity against *R. equi*, which could be further exploited for isolation of phytochemicals for treatment of foals or disinfection of stables.

Materials and Methods

Initial screening

In the present study, the research work was carried out 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 *Thespesia populnea* (Indian Tulip Tree / Paras Pipal), *Pongamia pinnata* (L.) Pierre (Karanj / Karanja), *Albizia lebbeck* (L.) Benth. (Siris / Woman's Tongue Tree), *Delonix regia* (Hook.) Raf. (Gulmohar / Flame Tree), *Cordia dichotoma* (Gunda / Lasoda) and *Dalbergia sissoo* (Sheesham / Shisham / Sisu / Tahli / Tali / Chirhol) were collected manually from campus of ICAR-NRCE-EPC, Jorbeer, Bikaner (Rajasthan), dried in hot air oven at 50 °C and grined in mixer grinder to powder formation. Prepared ethanolic extract [14] 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.

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 [22].

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.

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.

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*.

Evaluation of *in vitro* antibacterial activity

Disc diffusion method [28, 33] and agar well diffusion method [18] 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 < 10 mm diameter
Moderate Active – when IZ is > 10 mm and < 15 mm diameter
Good Active – when IZ is >15 mm diameter

Control

Azythromicin and rifampicin 10 mg/liter in ethanol were taken as control.

Polymerase Chain Reaction (PCR) Technique

Pure colony of *R. equi* was procured from National Center for Veterinary Type Cultures (NCVTC), National Research Center on Equine (NRCE), Hisar and verified time to time for purity by using the PCR technique [6]. We obtained the amplified 550 and 700 BP fragments of the *R. equi* pathogenic Vap A and Vap C genes respectively.

Results

Table 1. showing *in vitro* antibacterial activity of ethanolic, chloroformic, Sequentially Extracted Water Extract (SEWE) leaves extract of *Thespesia populnea* (Indian Tulip Tree / Paras Pipal), *Pongamia pinnata* (L.) Pierre (Karanj / Karanja), *Albizia lebbeck* (L.) Benth. (Siris / Woman's Tongue Tree), *Delonix regia* (Hook.) Raf. (Gulmohar / Flame Tree), *Cordia dichotoma* (Gunda / Lasoda) and *Dalbergia sissoo* (Sheesham / Shisham / Sisu / Tahli / Tali / Chirhol) against *R. equi*. On further polarity and solubility based fractionation, *in vitro* antibacterial activity against *R. equi* also showing in Table 1.

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

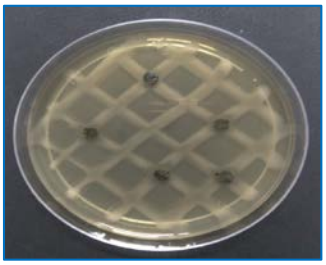
Plant	Part used	Extract / Fraction	Concentration	Method	Inhibition zone diameter	Degree of *in vitro* antibacterial activity
Thespesia populnea	Leaves	Ethanolic Extract	73.88 mg/ml	Disc Diffusion	Zero	None
Pongamia pinnata (L.) Pierre	Leaves	Ethanolic Extract	287.88 mg/ml	Disc Diffusion	Zero	None
Albizia lebbeck (L.) Benth.	Leaves	Ethanolic Extract	271.5 mg/ml	Disc Diffusion	Zero	None
Delonix regia (Hook.) Raf.	Leaves	Ethanolic Extract	145.85 mg/ml	Disc Diffusion	Zero	None
Cordia dichotoma	Leaves	Ethanolic Extract	313.8 mg/ml	Disc Diffusion	20.0 mm	Good
		Chloroformic Extract	201.67 mg/ml	Disc Diffusion	Zero	None
		SEWE	234.25 mg/ml	Agar Well Diffusion	Zero	None
Dalbergia sissoo	Leaves	Ethanolic Extract	128.28 mg/ml	Disc Diffusion	18.0 mm	Good
		Chloroformic Extract	158.0 mg/ml	Disc Diffusion	16.0 mm	Good
		SEWE	114.0 mg/ml	Agar Well Diffusion	Zero	None
		PESF of CE	8.12 mg/ml	Agar Well Diffusion	Zero	None
		EASF of CE	40.08 mg/ml	Agar Well Diffusion	Zero	None
		CSF of CE	28.86 mg/ml	Agar Well Diffusion	Zero	None
		ASF of CE	10.92 mg/ml	Agar Well Diffusion	Zero	None
		ESF of CE	35.8 mg/ml	Agar Well Diffusion	Zero	None

Leaves of *Thespesia populnea*

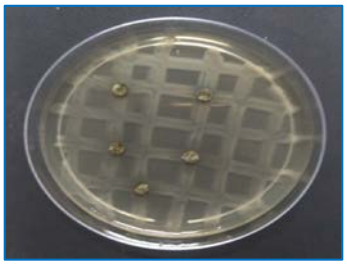
Leaves of *Pongamia pinnata* (L.) Pierre

Leaves of *Albizia lebbeck* (L.) Benth.

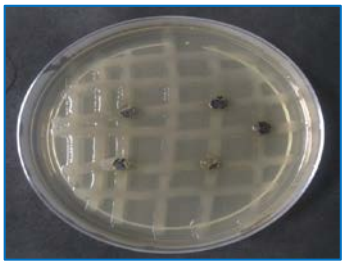
Leaves of *Delonix regia* (Hook.) Raf.



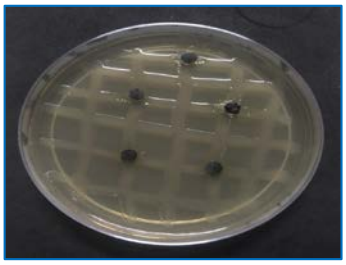
(a) Ethanolic Extract of *Thespesia populnea* leaves
IZD – 0.0 mm
Conc.– 73.88 mg/ml
(Disc Diffusion)



(b) Ethanolic Extract of *Pongamia pinnata* (L.) leaves
IZD – 0.0 mm
Conc. – 287.88 mg/ml
(Disc Diffusion)



(c) Ethanolic Extract of *Albizia lebbeck* (L.) leaves
IZD – 0.0 mm
Conc. – 271.5 mg/ml
(Disc Diffusion)

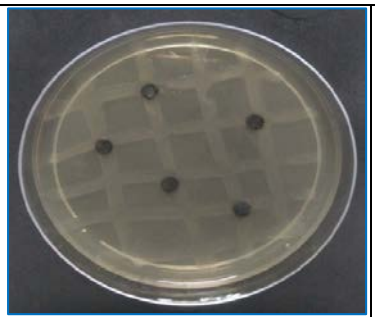


(d) Ethanolic Extract of *Delonix regia* (Hook.) leaves
IZD – 0.0 mm
Conc. – 145.85 mg/ml
(Disc Diffusion)

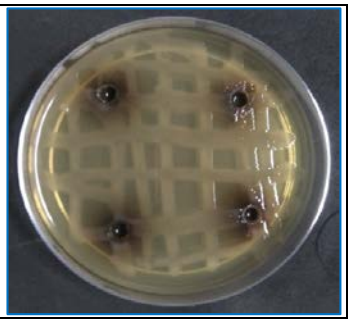
Fig 1: *In vitro* antibacterial activity of Ethanolic Extract of leaves of (a) *Thespesia populnea;* (b) *Pongamia pinnata* (L.) Pierre; (c) *Albizia lebbeck* (L.) Benth. and (d) *Delonix regia* (Hook.) Raf. against *R. equi*

Leaves of *Cordia dichotoma*

(a) Ethanolic Extract of leaves
IZD – 20.0 mm
Conc. – 313.8 mg/ml
(Disc Diffusion)

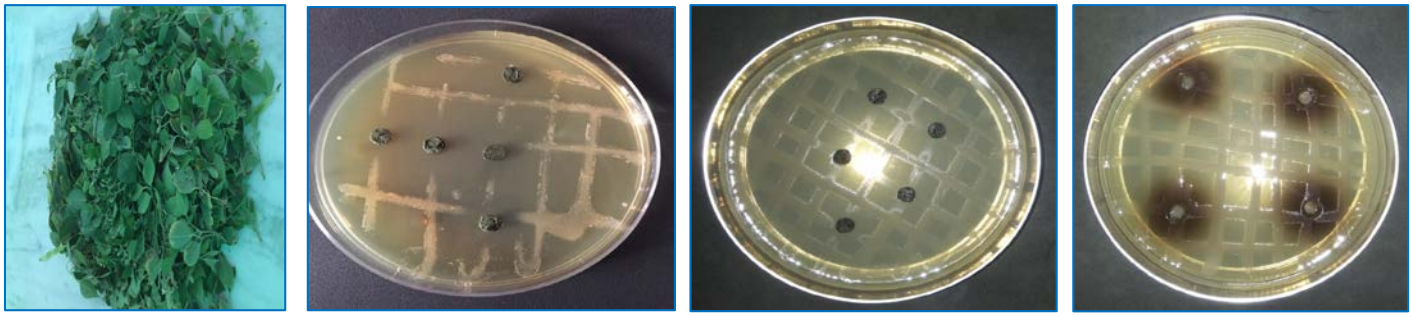


(b) Chloroform Extract of leaves
IZD – 0.0 mm
Conc. – 201.67 mg/ml
(Disc Diffusion)



(c) SEWE of leaves
IZD – 0.0 mm
Conc. – 234.25 mg/ml
(Agar Well Diffusion)

Fig 2: *In vitro* antibacterial activity of leaves extract of *Cordia dichotoma* against *R. equi*: (a) Ethanolic Extract (b) Chloroformic Extract (c) SEWE



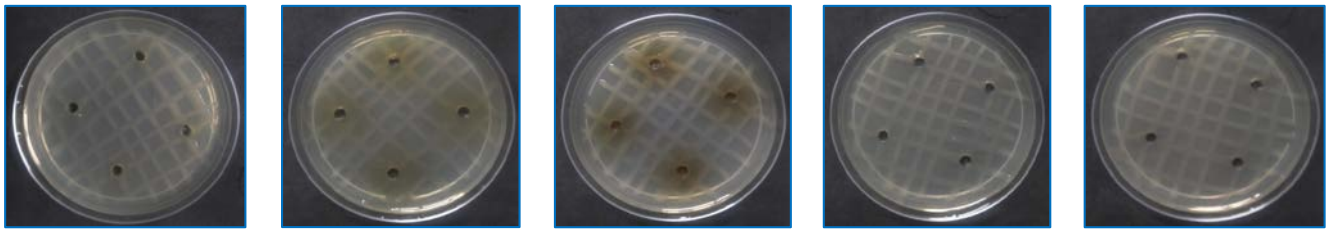
Leaves of *Dalbergia sissoo*

(a) Ethanolic Extract of leaves
IZD – 18.0 mm
Conc. – 128.28 mg/ml
(Disc Diffusion)

(b) Chloroform Extract of leaves
IZD – 16.0 mm
Conc. – 158.0 mg/ml
(Disc Diffusion)

(c) SEWE of leaves
IZD – 0.0 mm
Conc. – 114.0 mg/ml
(Agar Well Diffusion)

Fig 3: *In vitro* antibacterial activity of leaves extract of *Dalbergia sissoo* against *R. equi*: (a) Ethanolic Extract (b) Chloroformic Extract (c) SEWE



(a) PESF of CE
IZD – 0.0 mm
Conc. - 8.12 mg/ml
(Agar Well Diffusion)

(b) EASF of CE
IZD – 0.0 mm
Conc.-40.08 mg/ml
(Agar Well Diffusion)

(c) CSF of CE
IZD – 0.0 mm
Conc.-28.86 mg/ml
(Agar Well Diffusion)

(d) ASF of CE
IZD – 0.0 mm
Conc.-10.92 mg/ml
(Agar Well Diffusion)

(e) ESF of CE
IZD – 0.0 mm
Conc. - 35.8 mg/ml
(Agar Well Diffusion)

Fig 4: *In vitro* antibacterial activity of solvent based fractionation of Chloroformic Extract (CE) of *Dalbergia sissoo* leaves against *R. equi*: (a) PESF of CE (b) EASF of CE (c) CSF of CE (d) ASF of CE (e) ESF of CE

Discussion

R. equi colony

In the present study, the purity of *R. equi* colony verified time to time 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 [6].

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 ($C_2 H_5$) group. Chloroform dissolves non-polar compounds and distilled water dissolves polar compounds [14].

Non-active plants

In initial screening the ethanolic leaves extract of *Thespesia populnea* (Indian Tulip Tree / Paras Pipal), *Pongamia pinnata* (L.) Pierre (Karanj / Karanja), *Albizia lebbeck* (L.) Benth. (Siris / Woman's Tongue Tree) and *Delonix regia* (Hook.) Raf. (Gulmohar / Flame Tree) did not show *in vitro* antibacterial activity against *R. equi* (Fig.-1). There are so many factors like environment, pH of the medium, 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 plant extract [19].

Cordia dichotoma (Gunda / lasoda)

Most of the parts of this plant have been reported for having various medicinal properties [21]. Ethanolic extract of the plant at 314 mg/ml showed good *in vitro* antibacterial activity (20 mm diameter inhibition zone) against *R. equi* in disc diffusion method (Fig.2-a). So leaves extract of this plant was further sequentially fractionated in chloroform (CE) and water (sequentially extracted water extract, SEWE). Both the fractions have not shown further *in vitro* antibacterial activity (Fig.2-b and Fig.2-c), so it seems that the initial antimicrobial activity of ethanolic extract might have been shown by the combined effect of both polar and non-polar substances of the ethanolic extract (Fig.2-a). Similar to findings in present study, antimicrobial activity of *C. dichotoma* chloroform, methanol and aqueous extract did not show antimicrobial activity while acetone extract have shown antimicrobial activity against many gram positive and gram negative bacteria [29]. Since relative polarity of acetone (0.355) is more than chloroform (0.259), so it might be able to dilute some more polar substances along with non-polar substances like ethanol (0.654) in present study.

Dalbergia sissoo (*Sheesham* / *Shisham* / Sisu / Tahli / Tali / Chirhol)

Leaves of *D. sissoo* have antibacterial properties [31, 32]. In present study, ethanolic extract of the leaves of *D. sissoo* has shown good *in vitro* antibacterial activity (18 mm diameter inhibition zone at concentration 129 mg/ml) against *R. equi* (Fig.3-a). So it was sequentially fractioned in chloroform and then in water (sequentially extracted water extract, SEWE).

Chloroform extract (CE) has shown good *in vitro* antibacterial activity (16 mm diameter inhibition zone) at concentration of 158 mg/ml (Fig.3-b) while SEWE did not show *in vitro* antibacterial activity against *R. equi* (Fig.3-c). Further, fractions of chloroform extract in petroleum ether, ethyl acetate, chloroform, acetone and ethanol; no fraction was found having antimicrobial activity (Fig.-4). Possibly the active components were divided in different fractions so antimicrobial activity could not be observed. Active components of the *D. sissoo* were non-polar in nature and were soluble in chloroform. SEWE fraction also not shown *in vitro* antibacterial activity. Isolated chalcone showing antibacterial efficacy from hexane and methanolic extract of *D. sissoo* leaves [4].

Control - Azithromycin and Rifampicin

Azithromycin and Rifampicin were taken as control having concentration of 10 mg/liter and showed 25.0 mm (Fig.-5) and 20.0 mm (Fig.-6) diameter of inhibition zone respectively against *R. equi* using agar well diffusion method.

Fig 5: Control Azithromycin
IZ – 25.0 mm
conc. - 10.0 mg/liter
(Agar Well Diffusion)

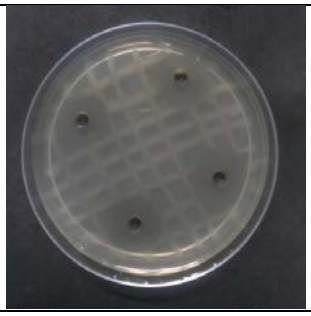


Fig 6: Control Rifampicin
IZ – 20.0 mm
Conc. - 10.0 mg/liter
(Agar Well Diffusion)

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 [7]. In present experiment, commercially available azythromicin and rifamipicin was used @ 10 mg/liter and both the antibiotics have shown good zone of inhibition (Fig.-5 and Fig.-6). While most effective herbal fraction chloroformic leaves extract of *Dalbergia sissoo* showed their minimum inhibitory concentration at 158 mg/ml. It shows that, quantitatively currently used antibiotics have more times antimicrobial efficacy than the fraction chloroformic leaves extract of *Dalbergia sissoo*. 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 chloroformic leaves extract of *Dalbergia sissoo* against *R. equi* as farm disinfectant.

Conclusion

On comparison with currently used antibiotics, required concentration of the most active chloroformic leaves extract of *Dalbergia sissoo* is too high for their possibilities for *in vivo* use. However, abundant availability of *Dalbergia sissoo* leaves and their antibacterial activity against *R. equi* suggests their potential for use as disinfectant against *R. equi* and further more investigation will be require phytochemical analysis for isolation of bioactive constituents responsible for their antimicrobial activity.

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Conflict of Interest

The authors declare no conflict of interest.

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