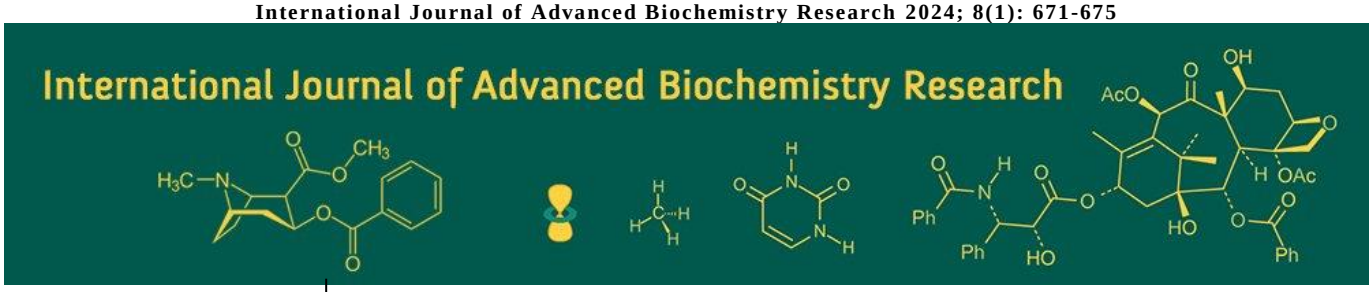

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In vitro antibacterial activity of leaves extracts of *bougainvillea* and *Ziziphus nummularia* against *Rhodococcus equi*

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Abstract
The experiment aimed to assess the *in vitro* bactericidal activity of ethanolic, chloroformic, and Sequentially Extracted Water Extract (SEWE) derived from the leaves of *Bougainvillea* and *Ziziphus nummularia* (*Burm. f.*) Wight & Arn. (also known as Jhar ber / jhar beri / wild jujube / Indian jujube). The ethanolic leaves extract of Bougainvillea and *Ziziphus nummularia* showed no action against positive *Rhodococcus equi* strains Vap A and Vap C in the initial screening conducted using the disc diffusion method. When compared to the antibiotics already in use, namely azithromycin and rifampicin, the ethanolic leaves extract of these plants did not exhibit any antibacterial action against *R. equi* in laboratory tests. Nevertheless, the ample abundance of plant leaves indicates the need for additional investigation on the antibacterial activity demonstrated *in vitro* using different solvents against *R. equi.*

Keywords: *Bougainvillea,* chloroform, ethanol, *In vitro, Rhodococcus equi* and *Ziziphus nummularia*

Introduction
Rhodococcus equi, a versatile gram-positive rod typically found in soil, is a notable pathogen that impacts young foals. R. equi infection results in the development of subacute or chronic bronchopneumonia characterized by the formation of abscesses, often accompanied with ulcerative typhlocolitis. The infection has the potential to spread and affect mesenteric lymphadenitis, osteomyelitis, purulent arthritis, reactive arthritis, and ulcerative lymphangitis (Dedar *et al*., 2017) [4]. R. equi is a major contributor to foal deaths, as around 17 to 20% of foals tested positive for *R. equi* in upper respiratory tract swab samples, according to studies conducted by Kumar *et al.* in 2014 [16] and Mir et al. in 2015 in Rajasthan and Jammu and Kashmir, respectively. *R. equi* is a facultative intracellular pathogen that thrives and reproduces within macrophages.
The condition is managed using a regimen that includes both rifampin and erythromycin, as described by Sweeney *et al*. (1987) [23] and Hillidge (1987) [10]. Recently, newer generation macrolides such as clarithromycin or azithromycin have replaced erythromycin in conjunction with rifampin, as highlighted by Gigue`re *et al.* in 2004 [8]. Strains resistant to either of these medications have also been found (McNeil and Brown, 1992; Fines *et al*., 2001; Kotze and Eloff, 2002; Asoh *et al*., 2003; Jacks *et al.*, 2003; Gigue`re *et al*., 2010 and Pauw and Eloff, 2014) [17, 6, 15, 1, 9, 20]. The rising usage of macrolides for disease control has been mentioned as a contributing cause to the emergence of resistance (Pauw and Eloff, 2014) [20]. The absence of effective alternatives against *R. equi* highlights the requirement to discover innovative antimicrobial medicines for managing and treating *R. equi* infections in foals. Plants display an outstanding capacity to create a varied array of secondary metabolites, including alkaloids, glycosides, terpenoids, saponins, steroids, flavonoids, tannins, quinones, and coumarins (Das *et al.*, 2010) [3]. These biomolecules serve as the foundation for plant-derived antibacterial compounds (Srivastava *et al.*, 2014) [22]. Certain natural compounds demonstrate high efficiency in the treatment of bacterial infections (Fernebro, 2011) [5].

(Jhar ber / jhar beri / wild jujube / Indian jujube) to identify So objective of The suggested investigation is designed to assess the *in vitro* antibacterial activity of leaf extracts of Bougainvillea and *Ziziphus nummularia* (Burm. f.) Wight & Arn. candidate plants having *in vitro* antimicrobial activity against *R. equi*.

Materials and Methods

(A) Method for the screening of *in vitro* antibacterial activity of ethanolic extract against *Rhodococcus equi:*

1. Plants and their parts have various bioactive chemicals, some of them are polar and some of them are non-polar. Ethanol is well known to dissolve both polar and non-polar molecules because of its polar character due to its hydroxyl group (OH-) and non-polar nature due to ethyl (C2 H5) group. So for the initial screening in the study plants's materials were extracted using ethanol by techniques reported by Gberikon *et al*., 2015 [7] with some changes and were submitted to antibacterial activity against R. equi using the procedures used by Gberikon *et al*., 2015 [7].

I. Collection of different parts of plant

- Fresh leaves of *Bougainvillea* and *Ziziphus nummularia* (Burm. f.) Wight & Arn. (Jhar Ber / Jhar Beri / Wild Jujube / Indian Jujube) were collected manually.

II. Drying of collected parts

1. Collected pieces were broken into little parts and spreaded in thin layer in the tray.
2. The tray was maintained in hot air oven at 50 ^{0}C for 24 to 48 hours for total dryness.

III. Preparation of powder:

1. Plant parts were grinded to make powder with the help of mixer grinder.
2. The powder was packed in the polythene bags and stored at room temperature for further use, preferably within a week.

IV. Preparation and storage of ethanolic extract:

i. Weighing and ethanol adding:

1. Exactly 50 gram of powder of each plant was taken in a borosil glass bottle of 1000 ml capacity.
2. Added absolute ethanol (99.9%), to fill the bottle.
3. It was kept overnight on shaking incubator at 37 ^{0}C.

ii. Sonication:

1. After overnight keeping, ethanolic solution with plant powder taken in to a clean dry glass beaker.
2. Sonicator (Model VCX 750, made Sonics & Materials, USA) was used for sonication on following conditions under ice.

a) Time ~ 20 minutes
b) Temperature ~ 60 ^{0}C
c) Pulse on ~ 59 seconds
d) Pulse off ~ 05 seconds
e) Amplitude ~ 70%

3. Sonicated extract was filtered in the glass flask by using whatman filter paper no.1.

iii. Rotary evaporation:

1. Filtrate was taken in the round bottom rotation bottle and it fixed at block nut of rotation motor.
2. Parameters of rotary evaporator machine were set as following:

a) Temperature of water bath ~ 60 ^{0}C
b) Temperature of chilling machine ~ 2 to 4 ^{0}C
c) Rotation ~ 70 to 100 rotations per minutes
d) Vacuum ~ 300 to 400 mmHg

3. Extract was collected when it started to stick on the wall of the flask.
4. Weight of the extract was taken against absolute ethanol in similar volume.

iv. Storage

Ethanolic e xtract was kept in the refrigerator at 4 to 5 ^{0}C temperature for further use within a week.

B. Polarity based fractionation of the active compound:

Plants whose ethanolic extracts were found efficient against *Rhodococcus equi* were exposed for further fractionation using chloroform and water sequential extraction to separate non-polar and polar components by adopting principles of procedures utilized by Jeyaseelan et al., 2012 [14].

C. Evaluation of *in vitro* antibacterial activity:

a) Disc diffusion method (Salie *et al*., 1996 and Nostro *et al*., 2000) [21, 19] was used for non-polar compounds and ethanolic extracts.
b) Agar well diffusion method (Irshad *et al*., 2012) [11] was used for polar compounds after dissolving the extract in sterile distilled water.
c) Pure colony of R. equi was purchased from National Center for Veterinary Type Cultures (NCVTC), National Research Center for Equine (NRCE), Hisar and tested time to time for purity by employing PCR based on pathogenic Vap A and Vap C genes.
d) Azythromicin and rifampicin 10 mg / liter in water were to taken as control.
e) Then keep it in the incubator at 37 ^{0}C temperature for 24 hours.

D. Observation of *in vitro* antibacterial activity of plant extracts:

1. After the incubation, observe the *in vitro* antibacterial activity of plant extract against the *Rhodococcus equi* by measuring the inhibition zone.
2. The diameter of inhibition zone to determine the degree of *in vitro* antibacterial activity of plant extract against the *R. equi* were followings:

a) Non-Active – inhibition zone not observed
b) Mild – when inhibition zone is < 10 mm diameter
c) Moderate – when inhibition zone is > 10 mm and < 15 mm diameter
d) Good – when inhibition zone is >15 mm diameter

Results

In this investigation, pure colony of R. equi was purchased from National Center for Veterinary Type Cultures (NCVTC), National Research Center on Equine (NRCE), Hisar and verified time to time for purity by using PCR based on pathogenic Vap A and Vap C genes. By the PCR approach, we achieved the ampilification of 550 and 700 bp fragements of the R. equi pathogenic Vap A and Vap C genes respectively. These pathogenic Vap A and Vap C genes demonstrated the colony of the R. equi was pure.

In the present investigation, leaves of Bougainvillea and *Ziziphus nummularia* (Burm. f.) Wight & Arn. (Jhar Ber / Jhar Beri / Wild Jujube / Indian Jujube) were taken for the screening of antibacterial activity against the *R. equi*. Ethanolic extracts of these plants were employed for *in vitro* antibacterial activity against *R. equi* and classed in four categories according to their activity as non-active, mildly active, moderately active and good active.

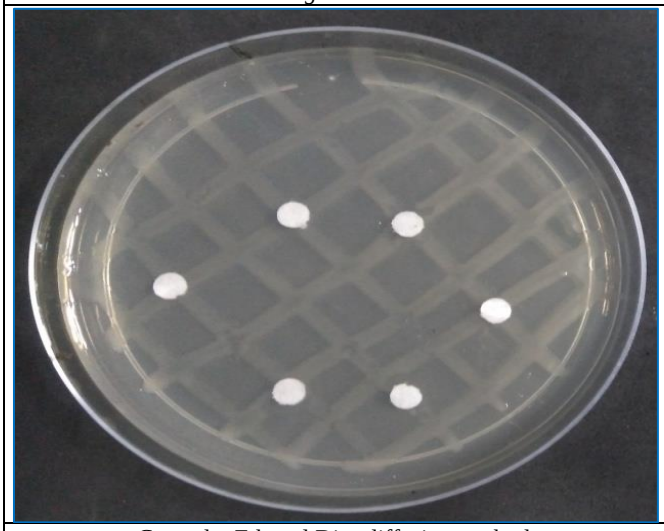
A. Plants showing no antibacterial activity (non-active) against *R. equi:*

1. *Bougainvillea* (*Bouganvilla*)

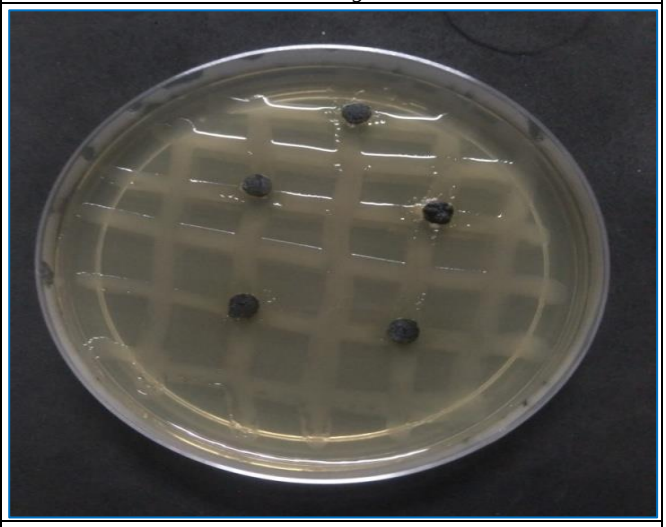
50 gm dry powder of leaves of Bougainvillea was used for manufacture of ethanolic extract, from which 8 ml of extract was obtained. The concentration of extract was 243.16 mg/ml. The extract did not show *in vitro* antibacterial activity against *R. equi* using disc diffusion technique. (Fig.1).

Bougainvillea

Leaves of *Bougainvillea*



Control – Ethanol Disc diffusion method



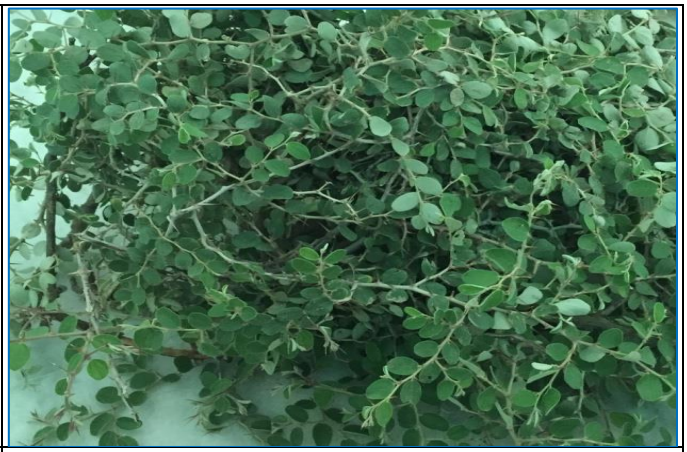
Ethanolic extract of leaves IZD – 0.0 mm; Conc. – 243.16 mg/ml

Fig 1: *Bougainvillea:* Ethanolic extract of leaves

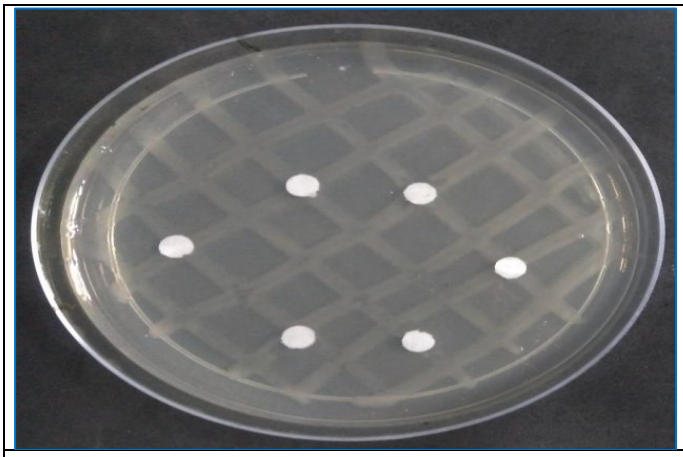
2. *Ziziphus nummularia* (Burm. f.) Wight & Arn. (Jhar ber / jhar beri / wild jujube / Indian jujube)

50 gm dry powder of leaves of *Ziziphus nummularia* (Burm. f.) Wight & Arn. was used for manufacture of ethanolic extract, from which 7.2 ml of extract was obtained. The concentration of extract was 259.09 mg/ml. The extract did not show *in vitro* antibacterial activity against *R. equi* using disc diffusion technique. (Fig. 2)

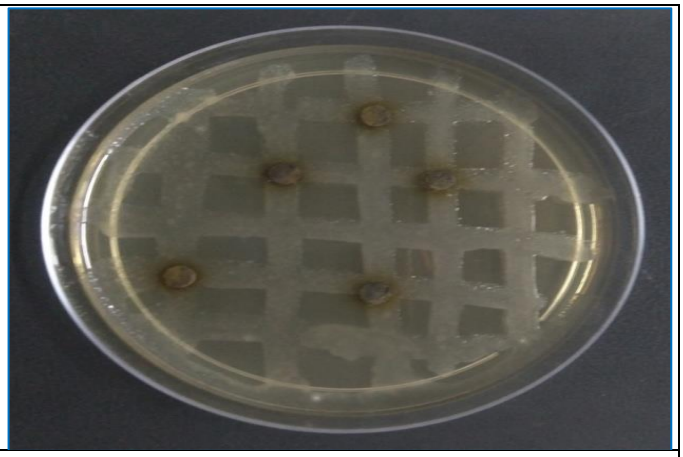
Ziziphus nummularia (Burm. f.) Wight & Arn.



Leaves of *Ziziphus nummularia* (Burm. f.) Wight & Arn.



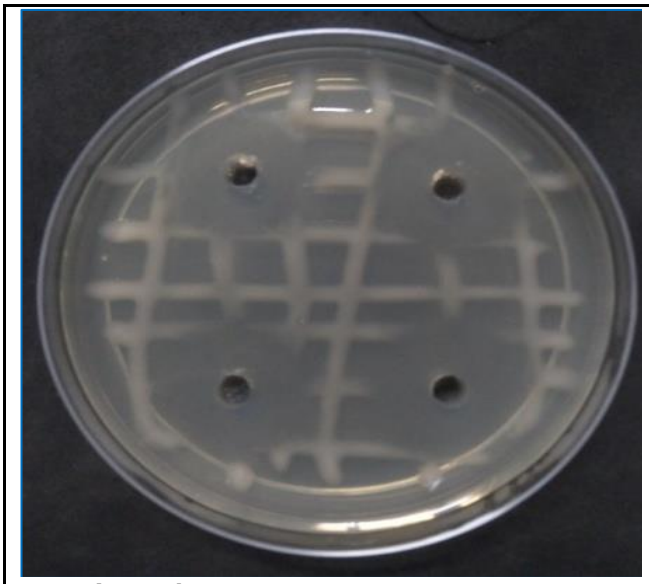
Control – Ethanol
Disc diffusion method



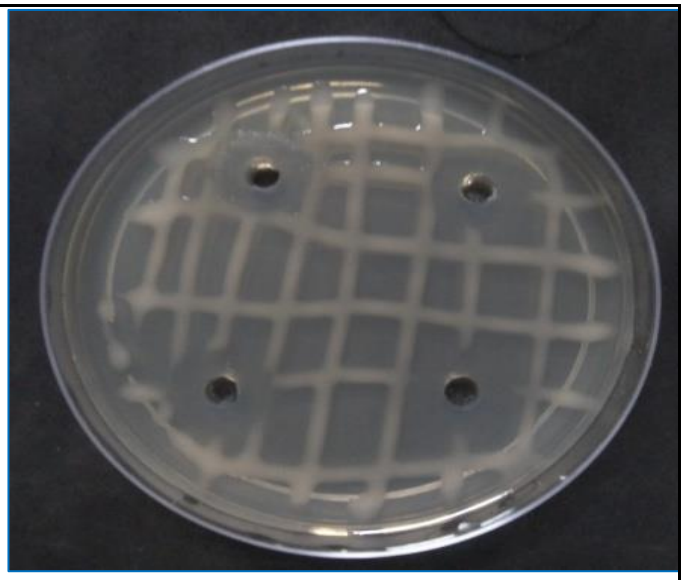
Ethanolic extract of leaves
IZD – 0.0 mm; Conc. – 259.09 mg/ml

Fig 2: *Ziziphus nummularia:* Ethanolic extract of leaves

B. Azithromycin and Rifampicin:
Azithromycin and Rifampicin were utilized as controls with a concentration of 10 mg/L, and they displayed inhibition zones of 25 mm and 20 mm in diameter, respectively, as assessed by the agar well diffusion method. (Fig. 3)



Control – Azithromycin IZD – 25 mm; Conc. - 10 mg/L



Control – Rifampicin IZD – 20 mm; Conc. - 10 mg/L

Fig 3: Control: Azithromycin and Rifampicin

Discussions
In the current work, the PCR approach indicated the amplification of 550 and 700 bp fragments matching to the pathogenic Vap A and Vap C genes of *R. equi*, respectively. The presence of two specific virulence-associated genes (Vap A and Vap C) showed the purity of the *R. equi* colony. The amplification targeted the nucleotide sequences of the Vap A gene (550 base pairs) and Vap C gene (700 base pairs) contained on the pathogenic plasmid. (Chhabra *et. al*., 2015).
The chemical solvents, ethyl alcohol (99.9%) and chloroform (99.9%), were utilized in their pure form. In the disc diffusion method, discs were immersed in various solvents and left to dry until total solvent evaporation happened. Consequently, the concentration of these chemical solvents in the dried discs was lowered to zero.

Plants showing no antibacterial activity (non-active) against *R. equi:* Ethanolic leaf extracts of *Bougainvillea* and *Ziziphus nummularia* (Burm. f.) Wight & Arn. (Jhar ber / jhar beri / wild jujube / Indian jujube) Demonstrated no *in vitro* antibacterial action against *R. equi*.
Numerous factors influence the antimicrobial susceptibility pattern of plant extracts, including environmental conditions, medium pH, temperature, water activity, oxygen availability, nutrient availability, choice of solvent, source of organisms, microbial biochemistry, physiology, metabolism, adaptation strategies, plant species, age, parts, concentration of the plant extract, and duration of extraction (Izah SC, 2018) [12].

Comparison with antibiotics
The combination of macrolides (erythromycin/azithromycin) and rifampicin is widely recognized as the most efficient treatment against R. equi in foals; nonetheless, resistance strains have been discovered (Cisek *et al.*, 2014) [2]. In the current experiment, commercially available azithromycin and rifampicin were

utilized at a dosage of 10 mg/L, and both antibiotics revealed a large zone of inhibition.

Conclusion

Ethanolic extracts of leaves of *Bougainvillea* and *Ziziphus nummularia* (Burm. f.) Wight & Arn. (Jhar ber / jhar beri / wild jujube / Indian jujube) Exhibited no *in vitro* antibacterial action against *R. equi*. However, significant availability of leaves of plants suggestes further additional investigation on their *in vitro* antibacterial activity with different solvents against *R. equi.*

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