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Exploration of Antimicrobial, Antibiofilm and Antiquorum Sensing Activity of the Himalayan Yellow Raspberry (*Rubus ellipticus*) against Clinical Isolates of *Escherichia coli* and *Staphylococcus aureus*

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ABSTRACT

Background: Management of ever growing multidrug resistant (MDR) bacteria becomes one of the biggest threats to public health worldwide. The situation is worsening due to lack of new generation antimicrobials in the arsenal of the clinicians. Development of new alternatives to the conventional antimicrobial agents is the need of the hour to control the menace of AMR. Plants based products are attractive alternatives with proven efficacy but needs scientific investigation to explore their potential antimicrobial, antibiofilm and antiquorum sensing activities against major bacterial pathogens of human and animals. The present study was conducted to explore the antimicrobial, antibiofilm and antiquorum sensing activity of aqueous and methanol extracts of leaf, flower, fruit and stem of the Himalayan yellow raspberry (*Rubus ellipticus*) against clinical isolates of *Staphylococcus aureus* and *Escherichia coli*.

Methods: *E. coli* and *S. aureus* were isolated and identified from diarrhoeic pigs and poultry and mastitic milk of cattle of Mizoram, respectively. Leaf, flower, fruit and stem/bark of *R. ellipticus* were collected from Mizoram and extracted by methanol and aqueous solvents. The antimicrobial activity and MIC was determined by well diffusion method and 96 wells microtiter plate method, respectively. Antibiofilm activity of plant extracts was determined in 96 well tissue culture plate. Antiquorum sensing activity was determined by disc diffusion method.

Result: Methanol leaf extract exhibited antimicrobial activity against *S. aureus* with 19 mm and 7 mm zone of inhibition at 200 mg/mL and 12.5 mg/mL, respectively. Methanol fruit extract also showed antimicrobial activity against *S. aureus* only. Highest and lowest activities were observed at 200 mg/mL and 25 mg/mL concentrations with 15 mm and 7 mm zone of inhibition, respectively. No antimicrobial activities by either of the extracts were observed against *E. coli* isolates. The MIC of *R. ellipticus* methanol leaf and fruit extracts against *S. aureus* was 0.203125 mg/mL and 0.8125 mg/mL, respectively. The methanol leaf (86.60%) and stem (85.60%) extracts of *R. ellipticus* showed significant antibiofilm activity against *S. aureus* isolates, whereas methanol fruit (89.20%) extracts exhibited antibiofilm activity against *E. coli* isolates at the concentration of 0.05 mg/mL. Significant antiquorum sensing (QS) activities was exhibited by the methanol leaf extract of *R. ellipticus* at 200 mg/mL concentration against *E. coli*. This is the first ever report on antibiofilm and anti QS activities of the *R. ellipticus* plant extracts against *E. coli* and *S. aureus* bacteria.

Key words: Antibiofim, Antimicrobial, Antiquorum sensing, *Rubus ellipticus*.

INTRODUCTION

Escherichia coli and *Staphylococcus aureus* are considered to be the major pathogens of human and animals associated with various disease conditions. Both the organisms are also posing serious threat due to ever growing antimicrobial resistance against existing antimicrobial agents. In addition, the biofilm producing multidrug resistant (MDR) pathogenic bacteria are considered as a major concern to public health. Majority of the antimicrobial drugs available in the arsenal of the medical and veterinary practitioners are becoming inactive (Dutta, 2020). The new generation antimicrobials are costly and limited for routine use for the common man in the middle and low income group of countries (Ghosh *et al*., 2019). With the huge threat posed by the MDR bacterial pathogens there is need to develop safe, dependable and cost effective alternatives to counter the menace of ever increasing threat of AMR.

Plant extracts and bioactive components isolated from ethnomedicinal plants are considered very important

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resources for novel antibacterial substances with various structures and mechanisms of action (Rios and Recio, 2005). There is increasing interest in the search for chemotherapeutic agents from ethnomedicinal plants used in traditional medicine (Sharma *et al*., 2010). *Rubus ellipticus*, also known as the yellow Himalayan raspberry is naturally found in forest edges over wide areas of mountains and lowlands of Sri Lanka and India especially the North eastern region of India (Wu *et al*., 2013). Due to their proven ethnomedicinal and pharmacological properties *Rubus* species has been widely used in folk medicine (Patel *et al*., 2004). In recent past, the phytochemical, antioxidant and medicinal attributes including the health promoting constituents of *Rubus ellipticus* are studied by various workers worldwide (Milivojevic *et al*., 2011; Wang and Lin 2000; Kafkas *et al*., 2008). Traditionally, the fruit of *R. ellipticus* is used in folk medicine for their astringent, febrifuge, kidney, miscellany and stomachic properties. The juice of the fruit is also used for treatment of fever, colic, coughs and sore throat (Pandey and Bhatt, 2016). The fruits of *R. ellipticus* are found to be highly nutritious, delicious, and rich in vitamins and sugars (Parmar and Kaushal, 1982). Although few sporadic reports are available on the antimicrobial activities of *R. ellipticus*, the antibiofilm and antiquorum sensing potentials of various parts of the plant remain unexplored. The present study was conducted to explore the efficacy of various extracts of different parts of *R. ellipticus* plant for their antimicrobial, antibiofilm and antiquorum sensing activities against clinical isolates of *E. coli* and *S. aureus*.

MATERIALS AND METHODS

Place and time of the work

The entire work was carried out during June, 2019 to July, 2020 at Department of Veterinary Microbiology, College of Veterinary Sciences and Animal Husbandry, Central Agricultural University, Selesih, Aizawl, Mizoram.

Bacterial culture

E. coli (n=50), isolated from fecal samples of diarrheic pigs and cloacal swabs of chickens and *S. aureus* (n=20), isolated from milk samples of mastitic cows of Aizawl, Mizoram were received from the repository of the Department of Veterinary Microbiology, College of Veterinary Sciences and Animal Husbandry, Central Agricultural University, Aizawl, Mizoram. All the bacteria were characterized by standard bacteriological techniques as described by Ewing (1986) and further confirmed by BD Phoenix automated bacterial system. *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) were used as control organisms under the study. All the pure bacterial isolates were stored at -80°C in glycerol (25% V/V) for further use.

Preparation of plant extract

Fresh leaf, flower, fruit and stem/bark of *R. ellipticus* plant (Fig 1) were collected from the campus of College of Veterinary Sciences and Animal Husbandry, Central Agricultural University, Selesih, Aizawl, Mizoram. Extraction of all the samples was done as per the method described by Elisha *et al*. (2017). In brief, all the components were washed with running tap water to remove the dust particles or other foreign bodies and air-dried under room temperature for several days until fully dried. The dried plant materials were ground individually to fine powder with a blender machine into coarse powder. All the coarse powder of root, stem, leaf and flowers was soaked independently in water, methanol and hexane solvents (1:10, w/v) for 5-6 h at room temperature. After soaking, the supernatant was collected and filtered through Whatman filter paper No. 1 followed by evaporation at 40°C under vacuum. The filtrate was concentrated in rotary vacuum evaporator (IKA, RV10 digital, Germany) and the concentrated extracts were re-suspended in the same solvents to make the final concentration @ 200 mg/mL. All the extracts were again filtered through 0.45 µm syringe filter and the filtrates were stored at -20°C till further use.

Antimicrobial activity of the plant extracts

The antimicrobial activity of the plant extracts was done by agar well diffusion method in Muller Hinton agar (HiMedia, Mumbai) as described by Lahlah *et al*. (2012). All the bacteria were grown on nutrient agar medium (HiMedia, Mumbai) and 2-3 pure colonies were picked up from the culture plate and transferred to the Luria Bertani (LB) broth. The tube was incubated for 4-5 hrs at 37°C and the inoculum density was standardized at 0.5 McFarland. The inoculums were inoculated over the MHA plate using absorbent cotton swab



Fig 1: Images of *Rubus ellipticus* (Yellow Himalyan raspberry) collected from College of Veterinary Sciences and Animal husbandry, Central Agricultural University, Selesih, Aizawl, Mizoram.

so that a lawn culture may grow. The holes of 6 to 8 mm diameter were made in the plate and were loaded with 20 µL of plant extracts in each well. All the plates were incubated at 37°C for 24 h. Diameters of zone of inhibition were measured by scale. Ciprofloxacin (5 µg) was used as positive control and *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) were used as control organisms.

Determination of minimum inhibitory concentration (MIC) of the plant extracts

MIC of the plant extracts were determined in 96 well plate following the method described by Mazzola *et al.* (2009), where 2,3,5 triphenyl tetrazolium chloride (TTC) was used as chromogenic agent. One hundred µL of LB broth was dispensed in each well of the 96 wells plate followed by 100 µL (20 mg) of each extracts added in the first well followed by serial two fold dilution of the plant extracts. Finally 50 µL of bacterial suspension (adjusted to 0.5 McFarland standard) were added to each well. Plates were covered and incubated at 37°C for 24 h. After incubation, 20 µL of 0.1% TTC were added to each well and incubated for 15 minutes. The MIC value was determined based upon the red coloration of the liquid in each wells.

Phenotypic determination of antibiofilm effect of plant extracts

The antibiofilm effect of the plant extracts was determined by tissue culture plate method (Sánchez *et al*., 2016). Overnight grown culture of bacteria (0.4 OD) was centrifuged at 7000 rpm for 10 min at 4°C. The cell pellet was washed twice with phosphate buffered solution (PBS) followed by centrifugation at 7000 rpm for 10 min at 4°C. Finally, the pellet was re-suspended in PBS and OD value was checked at 600 nm (0.4 OD) in spectrophotometer. A serial two fold dilution of the plant extracts was made followed by addition of 10 µL of 0.4 OD bacterial cultures in each wells and incubated at 37°C for 18-24 hours. Additional LB broth was added to make the final volume up to 200 µL in each wells and incubated at 37°C for 24 hrs. After incubation the wells were washed twice with PBS (pH 7.4) to remove free floating planktonic bacteria. Then 200 µL of 0.1% crystal violet solution was added in each well followed by incubation at 37°C for 30 min to stain the adhered cells. The wells were washed twice with 200 µL PBS (pH 7.4) to remove excess stain and the plates were air dried. Then 200 µL methanol was added in each wells to solubilize the bound crystal violet. The untreated wells were used as control (uninoculated broth and bacteria).

The OD value at 570 nm was recorded to check the result using the following formula:

$$\% \text{ Biofilm inhibition} = \frac{\text{OD Control-OD test}}{\text{OD Control}} \times 100$$

Where:
OD control is the absorbance of untreated control and OD test is the absorbance of treated.

Phenotypic determination of anti quorum sensing (QS) effect of plant extracts

The anti QS effect of the plant extracts was determined using the method described by Alvarez *et al*. (2012). Commercially available paper discs (6 mm in diameter) were soaked with various concentrations (200 mg/mL, 100 mg/mL, 50 mg/mL, 25 mg/ml and 12.5 mg/mL) of plant extracts and air dried under aseptic condition. All the discs were stored at refrigeration temperature till further use. *Chromobacterium violaceum* (ATCC12472) was used as known positive control bacteria and furanone (Sigma Aldrich) was used as known positive control agent to standardize the QS inhibition activities. One hundred µL (2.5×10^6 CFU/mL) of freshly prepared bacterial culture was plated over LB agar and allowed to air dry. Discs were placed on the plate (maximum 6 disks on 100 mm plate) at equal distance and incubated at 30°C for 18-24 hours. Disks containing normal saline solution (NSS) and furanone (100 µg) (Sigma) were used as negative and positive control, respectively. Zone of inhibition of pigment formation surrounding the disks was recorded manually by a scale. The result was classified based on the diameter of the zone of inhibition as follows: "not sensitive" for diameter less than 8 mm, "sensitive" between 9 and 14 mm, "very sensitive" between 15 and 19 mm and "extremely sensitive" for larger than 20 mm (Moreira *et al*., 2015).

RESULTS AND DISCUSSION

The *R. ellipticus* methanol leaf extract showed antimicrobial activity against *S. aureus* only. Highest and lowest activities were observed at 200 mg/mL and 12.5 mg/mL concentrations with 19 mm and 7 mm zone of inhibition, respectively. Similarly, the *R. ellipticus* methanol fruit extract showed antimicrobial activity against *S. aureus* only. Highest and lowest activities were observed at 200 mg/mL and 25 mg/mL concentrations with 15 mm and 7 mm zone of inhibition, respectively. No antimicrobial activities by either of the extracts were observed against *E. coli* isolates. Similar observations were also found in a study by Saklani *et al*. (2012) in Uttarakhand and Badhani *et al*. (2015) from North eastern India. The protective effect of *R. ellipticus* methanolic fruit extract is largely attributed to the presence of antioxidant phytochemicals such as polyphenolics, flavonoids, anthocyanins, carotenoids and vitamins in the fruits (Bhatt *et al*., 2013). The current trend of research suggested that *R. ellipticus* fruit or their extracts are rich in phenolics and anthocyanins and therefore, it is gaining special attention for their potential antioxidant activities also (Bhatt *et al*., 2013). Pantelidis *et al*. (2007) also reported that fruit of *R. ellipticus* constitute a good source of natural antioxidant substances and have been part of the human diet for centuries. The results of the present study are also in agreement with the observations of Lathar *et al*. (2015) from Tamil Nadu, India. This antimicrobial activity might be due to presence of bioactive compounds like alkaloids, phenols,

tannins, saponins, flavonoids, flavones, glycosids, carbohydrates, terpenes, triterpenes and proteins, which are significantly present in the methanol leaf extracts of *R. ellipticus* (Stanojevic *et al*., 2008). Flavonoids, the major group of phenolic compounds were also reported for their antimicrobial and antiviral activity earlier. Flavonoids are important for prevention of diseases associated with oxidative damage of the membrane, proteins and DNA. The plant methanolic extract exhibited a significant amount of total phenolic content and total flavenoid content compared to other extracts. The phenolic acids and flavonoids present in the plants are natural antioxidants and also have proved to antimicrobial activity (Stanojevic *et al*., 2008).

Our results indicated that Gram-positive bacteria were more responsive towards the *R. ellipticus* methanol extracts. Our observation is also in corroboration with the reports of Cushnie and Lamb (2005), who also reported that flavonoid compounds showed greater inhibition activities on Gram positive bacteria when compared to Gram-negative bacteria. Wang *et al*. (2008) also reported nearly similar observation against gram positive bacteria. High sensitivity of that particular extract against gram positive bacteria may be due to their cell wall and outer membrane structures. Gram negative bacteria carry an outer membrane and a unique periplasmic space, which inhibits either entry of the molecule or trapped (Shan *et al*., 2007). In the present study, the greater inhibition was observed with the leaf and fruit extract of *R. ellipticus*, which might be due to presence of different active compounds like kaempferol-3-O-β-D-glucoside, kaempferol and nar-ingenin (Susanti *et al.,* 2007). In addition, the results of the present study are also in corroboration with the observations of Alnajar *et al.* (2012) of Malaysia. Simanjuntak (2008) also reported the presence of flavonoids, saponins, tannins, glycosides, and steroids/ triterpenoids in the leaves of *M. malabathricum* collected from Sumatera, Indonesia.

In the present study, to determine the antimicrobial of medicinal plant extracts, agar well diffusion method and minimum inhibitory concentration (MIC) assays were used. The MIC was selected to test for antimicrobial activities of plant extracts, which provided quantitative results and is considered as the most appropriate and reliable method (Sigei *et al.,* 2015). *R. ellipticus* methanol leaf and fruit showed effective antimicrobial activity against *S. aureus* with the MIC value of 0.203125 mg/ml and 0.8125 mg/ml, respectively.

The methanol leaf and stem extracts of *R. ellipticus* showed significant antibiofilm activity against *S. aureus* isolates. Similarly, antibiofilm activity was also exhibited by methanol fruit extracts against *E. coli* isolates. Maximum inhibition was recorded with lowest dilution (0.05 mg/ml) of methanol leaf (86.60%), stem (85.60%) and fruit (89.20%) extracts of *R. ellipticus*. It has been observed that the antibiofilm potential of all the effective extracts were increased with increasing dilution and maximum activity was recorded with 0.05 mg/mL concentration, which might be due to the improved capacity of penetration of the molecules at lower concentration through the biofilm substances. Beyond 0.05 mg/mL concentration, the amount of active molecules was not at the threshold level to inhibit the biofilms. To the finest of our knowledge so far, no reports are available regarding the antibiofilm activity of any extracts from *R. ellipticus*. In related study in Universiti Kebangsaan Malaysia and Universiti Sains Islam Malaysia reported inhibition of biofilm formation against *Streptococcus mutans* by methanolic stem/bark extracts of *M. malabathricum* (Rohazila *et al*., 2015). Few bioactive compounds such as 8-metil-1-undecene, propanenitrile hexanoic acid and 1-decene have been recognized from sub-fraction 18 of the *M. malabathricum* stem bark, which could significantly lessen biofilm formation and adherence activity on *S. mutans* (Rohazila *et al*., 2015). Although the active principle of the crude extracts of *M. malabathricum* is not analyzed, it may be assumed that the antibiofilm activities recorded against *E. coli* and *S. aureus* were due to the similar compounds. The methanol extract of *Carex dimorpholepis* also exhibited antibiofilm properties against *E. coli* up to 78% at 0.10 mg/mL (Lee *et al*., 2013). Similarly, aqueous extracts of *Syzium leggati* could prevent the formation of biofilm by 72% at 0.05 mg/mL (Nostro *et al.,* 2016). In the present study, the antibiofilm activities of various extracts of *M. malabathricum* were more than 85.0% hence, proved to be the best so far. In addition, it is also the first ever reports on identification of various solvent extracts of different parts of *R. ellipticus* as potential biofilm inhibitor against *E. coli* and *S. aureus*. A low concentration of the plant extract may be required to prevent biofilm first attachment, while higher concentration of the plant extract is required to disrupt preformed biofilm (Stewart, 2002). Our study indicated that most plant extracts have the antibacterial coupled with antibiofilm activity; therefore, it may prove helpful for developing biofilm inhibitors and increase the effectiveness of infectious diseases treatment.

The methanol leaf extract of *R. ellipticus* exhibited good anti QS activities at 200 mg/mL concentration against *E. coli*. To the best of our knowledge no reports are available regarding the anti QS activity of *R. ellipticus* plant extracts and very limited reports are available even for the other plants. Anti QS activity against *E. coli* and *S. epidermidis* strains was recorded using *L. origanoides*, *Thymus vulgaris* and C*ymbopogon martini* oils (Pappenfort *et al*., 2017). The methanol leaf extract of *P. emblica* and flower extract of *M. indica* also exhibited broad spectrum anti QS activity, which affected the activity of acyl homoserine lactones and autoinducers over a wide range of sub-inhibitory concentrations (Zahin *et al*., 2010). Methanolic root extracts of *Hemidesmus indicus,* bark of *Holarrhena antidysenteri* and aqueous fruit extract of *Punica granatum* and leaf of *Mangifera indica* demonstrated varying level of AHL mediated violacein pigment inhibition in *Chromobacterium violaceium* (Nostro *et al.,* 2016). Simanksi *et al.* (2012) also reported that the *Vernonia amygdalina* methanol leaf extract

possessed anti QS activity against *S. aureus*. As an alternative approach to antibiotics, inactivating bacterial QS mechanisms is being widely studied. QS is a mechanism, through which bacterial cells can communicate with each other with the help of QS molecules, which can control the release of virulence determinants, bioluminescence, plasmid transfer, motility and biofilm formation. With the help of signaling molecules, called autoinducers, this system is controlled that pass through bacterial cell membranes. These signalling molecules are mostly synthesized by N-acyl-homoserine lactones (AHLs) in Gram-negative bacteria. In many studies it showed that plant secondary metabolites are responsible for QS inhibition because it can mimic QS molecules and in QS signalling pathways they inactivate their receptors, which is called quorum quenching (Doğan *et al*., 2019).

The antibiofilm and anti-QS are as important as the bacterial inhibition property to fight bacterial pathogenicity and studies enlarged in the last decade on this subject. The rise in antibiotic resistance of important pathogenic bacteria has also advanced these studies. This is the initial study about *R. ellipticus* from North eastern India for its antibacterial, antibiofilm and anti-QS effect against *E. coli* and *S. aurueus*. The methanol and aqueous extracts reduced the expression of biofilm production and QS activities of the target bacteria at very high level. In this regard, our study could contribute to discover new potential biofilm and QS inhibitor molecules against major pathogenic bacterial species. Future analysis on the extracts will possibly reveal novel bioactive compounds.

CONCLUSION

Methanol and aqueous solvent extracts of leaf, stem and fruit of *R. ellipticus* exhibited encouraging antimicrobial, antibiofilm and anti QS activities against clinical isolates of *E. coli* and *S. aureus* by *in vitro* techniques. This is the first ever report on antibiofilm and anti QS activities of the *R. ellipticus* plant extracts against *E. coli* and *S. aureus* bacteria. With the observations of the present study, it may be postulated that the extracts in its purified form may be used as topical antimicrobial preparation against biofilm producing bacterial agents.

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Conflict of interest

All authors declare that they have no conflict of interest.

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