Research Article

Assessment of antibacterial potential of methanol, n-hexane, ethyl acetate and chloroform *Moringa oleifera* leaf extracts

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Abstract
Bacterial infections and their increasing resistance to common antibiotics is posing serious threat to global public health. To this end, finding new alternatives and evaluating their antibacterial efficacy is always desirable. Therefore, in the present study we used methanol (MeOH), n-hexane (n-Hex), ethyl acetate (ETAC) and chloroform (CHCl₃) to prepare four different types of extracts from *Moringa oleifera* (*M. oleifera*) leaves aiming to inhibit five selected bacteria. Initially, agar well diffusion methods was used, and zones of inhibition were measured as an indicator of bacterial susceptibility. MeOH, n-Hex, ETAC and CHCl₃ leaf extracts showed highest zone of inhibition against *E. coli*, *B. cereus*, *S. pyogenes*, *S. aureus* and *S. enterica*, respectively. Moreover, highest zones of inhibition were observed at lowest incubation (24hr) and lowest zones were observed at highest incubation period (72hr) for all tested concentrations. Later, macrodilution method was used to access the antibacterial susceptibility in liquid medium. Results confirmed the susceptibility of all test bacteria with different level of IC₅₀ values ranging from 7.07 ± 0.44 to 10.91 ± 0.10 mg/ml for MeOH extract, 1.66 ± 0.08 to 2.11 ± 0.11 mg/ml for n-Hex extract, 2.58 ± 0.13 to 3.84 ± 0.21 mg/ml for ETAC extract and 3.73 ± 0.75 to 8.36 ± 0.20 mg/ml for CHCl₃ extract. Interestingly, none of the tested bacteria showed resistance against any of the tested extract in well diffusion or macrodilution method expressing the *M. oleifera* leaves extracts as potent candidates to kill bacteria in semisolid or in liquid medium to fulfill medical needs in future.

Keywords: Bacteria; *M. oleifera*; Macrodilution method; Zones of inhibition

Introduction
Antibiotics have been frequently used worldwide and saved countless lives. However, it is evident that the success of antibiotics might only be temporary, and we now expect a long-term or perhaps never-
ending challenge to find new effective alternatives to combat increasing problem of antibiotic resistance. The number of antibiotics such as trimethoprim, nitrofurantoin, fluoroquinolones, fosfomycin, gentamicin, sulfamethoxazole, cephalexin linezolid, telavancin daptomycin, vancomycin and clindamycin are easily available. However, bacterial resistance is reported to increase continuously at an alarming rate [1, 2]. For example, E. coli strains are found to be resistant to ampicillin, ciprofloxacin, cephalosporins, trimethoprim-sulfamethoxazole [3], S. aureus strains are found to be resistant to ampicillin, penicillin [4] and, B. cereus strains are found to be resistant to cephalosporin, penicillin. Resistance to clindamycin, cefazolin, cefotaxime and trimethoprim-sulfamethoxazole is also reported [5]. Moreover, S. enterica developed resistance to macrolides, aminoglycosides, cephalosporin, tetracycline, ketolides and ampicillin [6] and S. pyogenes strain developed resistance to most macrolides, fluoroquinolone macrolide, lincomamide and streptogramin antibiotics [7]. Therefore, search for new effective alternatives to combat microbial infections is always desirable.

Medicinal plants have been proven a good source of antibacterial therapy and have been used to treat diseases all over the world for many decades. Based on pharmacopoeias and study of medical plants in 91 countries, World Health Organization (WHO) reported almost 20,000 medicinal plant species [8]. Plants that inhibit the growth of microorganisms are important for human health and have been studied since 1926 [9, 10]. Herbal medicines being safe and environment friendly gained increasing popularity and are widespread around the globe encouraging to explore their biological properties to unmet the medical needs.

Thus, in the present study Moringa oleifera, one of the best known and widely distributed specie of a monogenic family Moringaceae [11] is selected for the evaluation of antibacterial properties. Selected plant ranges 5 to 10 m in height [12] and found wild near the sandy beds of rivers and streams or cultivated in plains, hedges and in-house yards. The numbers of medicinal properties have been associated to various parts of this highly esteemed tree. Plant parts including root, bark, leaf, fruit, gum, flowers and seed have been used for various ailments including infectious diseases, inflammation, gastrointestinal, cardiovascular, hematological and hepatorenal disorders [13, 14]. Moringa leaves are rich source of protein, vitamin C, β -carotene, calcium and potassium and have flavonoids, phenolics, carotenoids and ascorbic acid which serve as a valuable natural antioxidant and good food preserver [15, 13]. Interestingly, its leaves have been used to treat headaches, piles, sore throat, bronchitis, fevers, eye and ear infections, scurvy, diabetics as well as glandular swelling [12]. The juice from its root bark is used to put into ears to relieve earaches, placed in a tooth cavity as a pain killer and, has shown promising antitubercular activity [13]. Its seed extract showed protective effects by decreasing liver lipid peroxides.

Leaves of Moringa oleifera (M. oleifera) contain flavanoids and flavanol glycosides, glucosinolate and isothiocyanate, phenolic acid, alkaloid and sterol important for various biological activities including bacterial activity. Thus, in the present study we prepared methanol, n-hexane, ethyl acetate and chloroform M. oleifera leaf extracts and evaluated their antibacterial activity using five pathogenic bacterial strains.

**Materials and methods**

**Selected plant and preparation of extracts**

Moringa oleifera (M. oleifera) leaves were collected from a home garden, Mirpur, AJK, Pakistan. Selected plant part was washed 2-3 times under running tap water and air dried for 2 weeks under shade. Later, homogenized to fine powder and stored in airtight glass bottles for extract preparation. The powdered leaf sample was soaked in 1mg:10ml of methanol, n-hexane, ethyl acetate and chloroform in separate flasks for 5-7 days with 5 min shaking every day (Fig. 1). Later, filtrate was evaporated using rotary evaporator and air-dried concentrated extract was dissolved in respective solvent to prepare stock which were further diluted in broth to achieve desired concentration.
Figure 1. Different phases of *Moringa oleifera* leaf extracts

**Chemicals**
Methanol (MeOH), n-hexane (n-Hex), Chloroform (CHCl₃), Ethyl acetate (ETAC), 1, 1-di-phenyl-2-picryl hydrazyl (DPPH), analytical methanol, ethanol and ascorbic acid were used. Nutrient broth (Merck, Germany) and nutrient agar (OXOID CM003, UK) were used.

**Bacterial strains and culture conditions**
The bacterial strains *Bacillus cereus* (B. cereus) ATCC 10876, *Staphylococcus aureus* (S. aureus) ATCC 2592, *Streptococcus pyogenes* (S. pyogenes) ATCC 12384 and *Salmonella enterica* (S. enterica) were used in this study. Bacteria were streaked on agar plates, incubated at 37 °C for 17hrs. Later, plates were stored at 4 °C or used to prepare liquid culture in nutrient broth (Merck, Germany).

**Evaluation of antibacterial susceptibility by well diffusion method**
To evaluate the antibacterial effects of prepared extracts, well diffusion method was used [16]. Briefly, bacteria were spread homogeneously on agar plates. After short air dry, 5 wells (6mm in diameter) were made with the help of sterilized cork-borer. Then, each well was loaded with 50µl of 480 mg/ml, 240 mg/ml, 120 mg/ml, 60 mg/ml and 0 mg/ml (control) of test extract. Plates were incubated at 37 °C for 24hrs, 48hrs and 72hrs, photographed and zones of inhibition around the wells were measured in cm as an indicator of bacterial susceptibility.

**Testing bacterial inhibitory concentration (IC₅₀) by broth dilution method**
Bacterial inhibitory concentration of test extracts was determined by macro-broth dilution method. Serial dilutions of extract were placed in sample tubes having bacteria and, control tubes having broth only (no bacteria). The total volume per tube was adjusted as 1 ml with broth. Broth cultures were incubated at 37 °C for 24hrs. Later, IC₅₀ value in mg/ml was calculated for each extract against all test bacteria to compare effects in liquid medium.

**Statistical analysis**
All experiments were performed three times in triplet. Student’s t-test was applied to check significant difference using lowest zone of inhibition (except zero) vs each tested zone of inhibition (²p<0.05; ³p<0.005; ⁴p<0.0001) [17].

**Results and discussion**

**Antibacterial susceptibility**
In the present study different *M. oleifera* leaves extracts were prepared using methanol, n-hexane, chloroform and ethyl acetate. Later, their antibacterial effect against *B. cereus*, *E. coli*, *S. enterica*, *S. aureus* and *S. pyogenes* was compared in concentration dependent and incubation dependent manners.
Antibacterial effect of methanolic leaf extract
The tested methanolic (MeOH) leaf extract concentrations i.e. 60 mg/ml, 120 mg/ml, 240 mg/ml and 480 mg/ml showed 0cm, 0cm, 0.09cm, 0.15cm zones of inhibition against B. cereus, 0cm, 0cm, 0.13cm, 0.22cm zones of inhibition against S. aureus, 0cm, 0cm, 0.09cm, 0.17cm zones of inhibition against S. pyogenes, 0cm, 0cm, 0.1cm, 0.15cm zones of inhibition against S. enterica and 0.01cm, 0.1cm, 0.18cm, 0.3cm zones of the inhibition against E. coli, respectively at 24hr incubation (Table 1). Thus, bacterial susceptibility trend observed at highest tested concentration (480mg/ml) at 24hr incubation was as E. coli > S. aureus > S. pyogenes > S. enterica > B. cereus. Interestingly, highest zones of inhibition were observed at 24hr than 72hr incubation at all tested concentrations as shown in Table 1.

Antibacterial effect of n-hexane leaf extract
The tested n-hexane (n-Hex) leaf extract concentrations i.e. 60 mg/ml, 120 mg/ml, 240 mg/ml and 480 mg/ml showed 0.45cm, 0.5cm, 0.6cm, 0.61cm zones of inhibition against B. cereus, 0.42cm, 0.48cm, 0.5cm, 0.57cm zones of inhibition against S. aureus, 0.37cm, 0.44cm, 0.47cm, 0.49cm zones of inhibition against S. pyogenes, 0.32cm, 0.36cm, 0.44cm, 0.53cm zones of inhibition against S. enterica and 0.31cm, 0.34cm, 0.41cm, 0.47cm zones of the inhibition against E. coli, respectively at 24hr incubation (Table 1). Thus, bacterial susceptibility trend observed at highest tested concentration (480mg/ml) at 24hr incubation was as B. cereus > S. aureus > S. enterica > S. pyogenes > E. coli. Interestingly, highest zones of inhibition were observed at 24hr than 72hr incubation at all tested concentrations as shown in Table 1.

Antibacterial effect of ethyl acetate leaf extract
The tested ethyl acetate (ETAC) leaf extract concentrations i.e. 60mg/ml, 120mg/ml, 240mg/ml and 480mg/ml showed 0.28cm, 0.33cm, 0.38cm, 0.40cm zones of inhibition against B. cereus, 0.32cm, 0.38cm, 0.43cm, 0.47cm zones of inhibition against S. aureus, 0.37cm, 0.39cm, 0.45cm, 0.50cm zones of inhibition against S. pyogenes, 0.27cm, 0.35cm, 0.40cm, 0.46cm zones of inhibition against S. enterica and 0.27cm, 0.34cm, 0.40cm, 0.43cm zones of the inhibition against E. coli, respectively at 24hr incubation (Table 1). Thus, bacterial susceptibility trend observed at highest tested concentration (480mg/ml) at 24hr incubation was as S. pyogenes > S. aureus > S. enterica > E. coli > B. cereus. Interestingly, highest zones of inhibition were observed at 24hr than 72hr incubation at all tested concentrations as shown in Table 1.

Antibacterial effect of chloroform leaf extract
The tested chloroform (CHCl3) leaf extract concentrations i.e. 60 mg/ml, 120 mg/ml, 240 mg/ml and 480 mg/ml showed 0.19cm, 0.21cm, 0.24cm, 0.27cm zones of inhibition against B. cereus, 0.10cm, 0.18cm, 0.24cm, 0.28cm zones of inhibition against S. aureus, 0.15cm, 0.21cm, 0.26cm, 0.29cm zones of inhibition against S. pyogenes, 0.18cm, 0.26cm, 0.31cm, 0.38cm zones of inhibition against S. enterica and 0.13cm, 0.16cm, 0.24cm, 0.30cm zones of the inhibition against E. coli, respectively at 24hr incubation (Table 1). Thus, bacterial susceptibility trend observed at highest tested concentration (480mg/ml) at 24hr incubation was as S. enterica > E. coli > S. pyogenes > S. aureus > B. cereus. Interestingly, highest zones of inhibition were observed at 24hr than 72hr incubation at all tested concentrations as shown in Table 1.

M. oliefera, chloroform root bark extract contains aglycon of deoxy-niazimicin (N-benzyl, S-ethyl thioformate) which strongly inhibit C. albicans, P. aeruginosa, S. aureus, S. dysenteriae, S. boydii and S. typhi [18, 14]. Ethyl acetate bark extract of M. oliefera is reported to show higher inhibition toward Staphylococcus aureus, Pseudomonas fluorescens, Citrobacter freundii and Bacillus megaterium than methanol, chloroform, and aqueous extracts of the same part of the plant [19].
Table 1. Measured zones of bacterial inhibition by various *M. oleifera* leaves extracts.

| Test | Extract type | Concentration (mg/ml) | Zone of inhibition (cm) at 24 hr incubation | Zone of inhibition (cm) at 48hr incubation | Zone of inhibition (cm) at 72hr incubation | Concentration (mg/ml) | TEST | Zone of inhibition (cm) at 24 hr incubation | Zone of inhibition (cm) at 48hr incubation | Zone of inhibition (cm) at 72hr incubation |
|------|--------------|-----------------------|---------------------------------------------|--------------------------------------------|--------------------------------------------|----------------------|------|---------------------------------------------|--------------------------------------------|--------------------------------------------|
|      |              | 60                    | 120                                         | 240                                         | 480                                         | 60                   | 120  | 240                                         | 480                                         | 60                                         |
|      | MeOH         | 0.0±0.00              | 0.0±0.00                                    | 0.09±0.02                                   | 0.15±0.03b                                  | 0.00±0.00            | 0.00±0.00            | 0.08±0.01                             | 0.14±0.04b                                  | 0.00±0.00                                  |
|      | n-Hex        | 0.45±0.03             | 0.5±0.02                                    | 0.60±0.03                                   | 0.61±0.03                                   | 0.44±0.01            | 0.49±0.02            | 0.56±0.02                             | 0.60±0.01                                   | 0.43±0.04                                  |
|      | CHCl3        | 0.28±0.01             | 0.33±0.03                                   | 0.38±0.01a                                  | 0.40±0.02b                                  | 0.25±0.03            | 0.31±0.04            | 0.38±0.02b                             | 0.39±0.02b                                  | 0.22±0.02                                  |
|      |              | 0.19±0.09             | 0.21±0.02                                   | 0.24±0.03                                   | 0.27±0.02a                                  | 0.18±0.02            | 0.20±0.02            | 0.23±0.02                             | 0.25±0.02                                  | 0.15±0.03                                  |
|      | MeOH         | 0.0±0.00              | 0.0±0.00                                    | 0.13±0.01                                   | 0.22±0.02b                                  | 0.00±0.00            | 0.00±0.00            | 0.12±0.02                             | 0.20±0.04b                                  | 0.0±0.00                                   |
|      | n-Hex        | 0.42±0.04             | 0.48±0.03                                   | 0.50±0.04                                   | 0.57±0.03                                   | 0.40±0.03            | 0.46±0.03            | 0.48±0.03                             | 0.55±0.02                                   | 0.39±0.02                                  |
|      | ETAC         | 0.32±0.06             | 0.38±0.03a                                  | 0.43±0.03b                                  | 0.47±0.08b                                  | 0.29±0.03            | 0.36±0.02            | 0.41±0.03b                             | 0.46±0.02b                                  | 0.26±0.02                                  |
|      | CHCl3        | 0.32±0.06             | 0.38±0.03a                                  | 0.43±0.03b                                  | 0.47±0.08b                                  | 0.29±0.03            | 0.36±0.02            | 0.41±0.03b                             | 0.46±0.02b                                  | 0.26±0.02                                  |
|      | MeOH         | 0.0±0.00              | 0.0±0.00                                    | 0.09±0.00                                   | 0.17±0.02b                                  | 0.00±0.00            | 0.00±0.00            | 0.09±0.02                             | 0.17±0.05b                                  | 0.00±0.00                                  |
|      | n-Hex        | 0.37±0.03             | 0.44±0.03                                   | 0.47±0.04                                   | 0.49±0.05b                                  | 0.36±0.02            | 0.42±0.02            | 0.46±0.02                             | 0.48±0.02b                                  | 0.33±0.06                                  |
|      | ETAC         | 0.37±0.02             | 0.39±0.02                                   | 0.45±0.04a                                  | 0.50±0.03b                                  | 0.32±0.02            | 0.38±0.02            | 0.43±0.02a                             | 0.47±0.03b                                  | 0.29±0.02                                  |
|      | CHCl3        | 0.15±0.01             | 0.21±0.02                                   | 0.26±0.02a                                  | 0.29±0.02b                                  | 0.13±0.02            | 0.20±0.02            | 0.23±0.02                             | 0.27±0.02b                                  | 0.11±0.02                                  |
|      | MeOH         | 0.0±0.00              | 0.0±0.00                                    | 0.1±0.00                                    | 0.15±0.02a                                  | 0.0±0.00             | 0.0±0.00             | 0.0±0.00                              | 0.14±0.02a                                  | 0.0±0.00                                   |
|      | n-Hex        | 0.32±0.00             | 0.36±0.02                                   | 0.44±0.04b                                  | 0.53±0.03b                                  | 0.3±0.00             | 0.33±0.02            | 0.43±0.03b                             | 0.51±0.03b                                  | 0.29±0.01                                  |
|      | ETAC         | 0.27±0.08             | 0.35±0.01                                   | 0.40±0.03b                                  | 0.46±0.02b                                  | 0.24±0.03            | 0.32±0.04            | 0.38±0.02b                             | 0.43±0.03b                                  | 0.21±0.04                                  |
|      | CHCl3        | 0.18±0.00             | 0.26±0.03                                   | 0.31±0.04a                                  | 0.38±0.03b                                  | 0.15±0.01            | 0.25±0.03            | 0.29±0.04a                             | 0.35±0.02a                                  | 0.13±0.04                                  |
|      | MeOH         | 0.01±0.00             | 0.17±0.01                                   | 0.18±0.02b                                  | 0.30±0.02b                                  | 0.00±0.00            | 0.09±0.02            | 0.16±0.04a                             | 0.27±0.05a                                  | 0.00±0.00                                  |
|      | n-Hex        | 0.31±0.01             | 0.34±0.02                                   | 0.41±0.03                                   | 0.47±0.03                                   | 0.29±0.00            | 0.33±0.02            | 0.40±0.02                             | 0.46±0.02                                   | 0.25±0.00                                  |
|      | ETAC         | 0.27±0.01             | 0.34±0.04a                                  | 0.40±0.03b                                  | 0.43±0.02b                                  | 0.22±0.02            | 0.32±0.03            | 0.37±0.04a                             | 0.42±0.02b                                  | 0.19±0.04                                  |
|      | CHCl3        | 0.13±0.01             | 0.16±0.08                                   | 0.24±0.02b                                  | 0.3±0.02a                                   | 0.12±0.02            | 0.15±0.08            | 0.23±0.01b                             | 0.27±0.02b                                  | 0.09±0.01                                  |

Note: *a, b, c* indicate significant differences at the 0.05, 0.01, and 0.001 levels, respectively.
TABLE 2. Determination of bacterial inhibitory concentration (IC$_{50}$) by broth macrodilution method

| Extract type | Bacterial inhibition IC$_{50}$± SEM (mg/ml) | B. cereus | S. aureus | S. enterica | S. pyogenes | E. coli |
|--------------|---------------------------------------------|----------|----------|------------|------------|--------|
| MeOH extract | 9.68 ± 0.29                                 | 10.91 ± 0.10 | 9.02 ± 0.00 | 8.16 ± 0.00 | 7.07 ± 0.44 |
| n-Hex extract| 1.78 ± 0.00                                 | 1.70 ± 0.04 | 2.11 ± 0.11 | 1.66 ± 0.08 | 1.74 ± 0.10 |
| ETAC extract | 3.84 ± 0.21                                 | 2.58 ± 0.13 | 3.54 ± 0.05 | 3.50 ± 0.02 | 2.73 ± 0.27 |
| CHCl$_3$ extract | 4.44 ± 0.13                            | 4.34 ± 0.97 | 3.73 ± 0.75 | 8.36 ± 0.20 | 7.20 ± 0.17 |

However, in the present study each leaf extract exhibited different levels of antibacterial effects depending upon test bacteria. Methanol, n-hexane, ethyl acetate and chloroform leaf extracts showed highest zone of inhibition against *E. coli*, *B. cereus*, *S. pyogenes*, *S. aureus* and *S. enterica*, respectively. Interestingly, highest zones of inhibition were observed at lowest incubation (24hr) and lowest zones were observed at 72hr at all tested concentrations which is in accordance to our previous findings [17].

**Testing bacterial inhibitory concentration (IC$_{50}$) by broth dilution method**

To evaluate bacterial susceptibility in suspension state, calorimetric method was used. The extract concentration required to achieve 50% bacterial inhibition is expressed as inhibitory concentration (IC$_{50}$). Methanolic leaves extract showed IC$_{50}$ values as 9.68 mg/ml, 10.91 mg/ml, 7.07 mg/ml, 8.16 mg/ml and 7.07 mg/ml against *B. cereus*, *S. aureus*, *E. coli*, *S. pyogenes* and *S. enterica*, respectively (Table 2). The IC$_{50}$ trend for methanolic leaves extract was observed as *E. coli* > *S. pyogenes* > *S. enterica* > *B. cereus* > *S. aureus*. The n-hexane leaves extract showed IC$_{50}$ values as 1.78 mg/ml, 1.70 mg/ml, 1.74 mg/ml, 1.66 mg/ml and 2.11 mg/ml against *B. cereus*, *S. aureus*, *E. coli*, *S. pyogenes* and *S. enterica*, respectively. The IC$_{50}$ trend of n-hexane extract was *S. pyogenes* > *S. aureus* > *E. coli* > *B. cereus* > *S. enterica*. Ethyl acetate leaves extract showed IC$_{50}$ values as 3.84 mg/ml, 2.58 mg/ml, 2.73 mg/ml, 3.50 mg/ml and 3.54 mg/ml against *B. cereus*, *S. aureus*, *E. coli*, *S. pyogenes* and *S. enterica*, respectively. The IC$_{50}$ trend of ethyl acetate extract was *S. aureus* > *E. coli* > *S. pyogenes* > *S. enterica* > *B. cereus*. Moreover, chloroform leaves extract showed IC$_{50}$ values as 4.44 mg/ml, 4.34 mg/ml, 7.20 mg/ml, 8.36 mg/ml and 3.73 mg/ml against *B. cereus*, *S. aureus*, *E. coli*, *S. pyogenes* and *S. enterica*, respectively. The IC$_{50}$ trend of chloroform leaves was *S. enterica* > *S. aureus* > *B. cereus* > *E. coli* > *S. pyogenes* (Table 2). Kaur et al [20] reported that a methanolic leaves extract and 70% ethanolic extract of *M. oleifera* showed antileishmanial activity against *L. donovani* promastigotes. The ethyl acetate fraction of a methanolic extract was reported to show leishmaniasis with an IC$_{50}$ of 27.5 µg/ml. Moreover, *M. oleifera* extracts are reported to exhibit antiviral, antifungal, antihyperglycemic, antihyperlipidemic, and hypocholesterolemic activities. However, in the present study methanol, n-hexane, ethyl acetate and chloroform leaf extracts showed antibacterial effect against all five tested bacteria. Recently, bacteria including *B. cereus*, *S. aureus*, *S. pyogenes*, *S. enterica* and *E. coli* are reported to show resistance to commonly used antibiotics at alarming rate [1-7]. However, none of the tested bacteria showed resistance against any of the tested extract in well diffusion or
macrodilution method expressing the M. oleifera leaves extracts as potent candidates to kill bacteria in semisolid or in liquid medium for bacterial inhibition.

Conclusion
In the present study four extracts from M. oleifera leaves were prepared using methanol, n-hexane, chloroform and ethyl acetate. All extracts expressed bacterial inhibition against all tested bacteria i.e. B. cereus, E. coli, S. enterica, S. aureus and S. pyogenes in both well diffusion (semi solid medium) and macrodilution method (liquid medium) presenting the M. oleifera leaves extracts as potent candidates to treat tested bacteria in future.

Authors’ contributions
Conceived and designed the experiments: A Ali & N Rafiq, Performed the experiments: N Rafiq, Analyzed the data: H Khurshid, B Akbar, Z H Tarar, Contributed materials/ analysis/ tools: I Ahmed, F Nazir & M Ahmed, Wrote the paper: A Ali, N Rafiq & H Javed.

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