ABSTRACT

Antibiotic resistance is a dire public health problem, throughout the world, exclusively in developing countries. Moreover, south Asian part including Pakistan is considered to be the prime territory for antibiotic drug resistance. This immense threat and challenge elevated by multiple antibiotic resistance could be promptly handled by the development and / discovery of advance antibacterial agents from natural sources. For this aim, the current study focused on earthworm *Pheretima posthuma* as a model organism to evaluate *P. posthuma* coelomic fluid (PCF) and body paste (PBP) as antibacterial, antibiofilm and anti-quorum sensing (QS) agent.

During this study, total 616 earthworm's specimen were collected from eight (8) regions of Maralah Ravi Link canal in Sialkot district during the year 2019. Further, 276 juvenile worms were separated using hand sorting method from 342 adults. Results showed the highest number (105) of earthworms were collected from Propi Nagra Village (PNV19), while the lowest number (69) of specimens was collected from Jamkay Cheema Bridge (JCB19) region. Moreover, 86 pheretimoid earthworm species were isolated from 342 adult earthworms by examining different morphological characteristics like skin colour, number of segments, position of dorsal pores, clitellum position, setae and shape of prostomium. Results revealed that among 86 morphologically distinct earthworms, three Pheretimoid genera were observed. In total, 56 earthworm species were related to *Metaphire*, 28 to *Amynthas* and 02 to *Pheretima*. These identified genera consist of 18 belonging to *Metaphire posthuma*, 16 to *Metaphire houlleti*, 12 to *Metaphire birmanica,* 08 to *M. californica*, 02 to *M. anomala*, 11 to *Amynthas morrisi*, 11 to *A. minimus*, 03 to *A. diffringens*, 02 to *A. gracilis*, 01 to *Amynthas agrestis* 02 to *Pheretima lignicola*. From the results, it was concluded that *M. posthuma* species was the most abundant (28%) and diverse (found at all sampling sites, except JCB19) for member of Pheretimoid earthworm complex.

Molecular identification, phylogenetic relationships and evolutionary divergence time of earthworms belonging to the pheretimoid complex were investigated in this study using partial mitochondrial *COI* (cytochrome C oxidase subunit I) gene sequences. From 86 morphologically identified earthworms, 11 pheretimoid species were molecularly confirmed by DNA barcoding, such as *M. posthuma* (02), *M. birmanica*

(02), *M. houlleti* (02), *M. anomala* (01), *M. bununa* (01), *M. californica* (01), *A. morrisi* (01) and *A. minimus* (01). As it was the first attempt in Pakistan, this study has provided the genomic stamp to the earthworms that were identified through DNA barcoding.

Further, a phylogenetic tree was constructed through Maximum likelihood method by using 12 partial *COI* gene sequences and 48 GenBank adapted sequences along with *Hirudo medicinalis* (HQ33518.1), a non-earthworm annelid, as an outgroup. A monophyly of two well-supported lineages, a simple (A) and complex (B), with bootstrap values of 95% was developed. Similarly, a clade E formed a monophyletic tree which include *M. posthuma* (4). *M. birmanica* (3) *M. grandipenes* (2) and *M. houlleti* (1) with 100% bootstrap support. From the results, it was also concluded that the mono-phylogeny of identified species showed overall similarity with adapted similar NCBI species. During divergence time estimation, the phylogenetic lineage indicated that the members of two major groups of phylum Annelida, such as the *Pheretimoid* sp. (Megascolecidae family) and *H. medicinalis* (an outgroup) diversified 13 Mya (Million years ago) in Cenozoic Era (16.4 to11.2 Mya). While, pheretimoid complex species were continually diverged 5 times in Pliocene epoch (5.3 to 2.4 Mya) and 7 times in the Miocene epoch (11.2 to 5.3 Mya). During diversification, new genera like *Metaphire*, *Pheretima* and *Amynthas* were developed. From time study, it was concluded from chronogram that all the evolutionary events of earthworm occurred in Cenozoic era.

Following above, pairwise genetic distances were calibrated among intra or inter species by p-distance comparison to corroborate the taxonomic status of pheretimoid earthworms. The pairwise p-distance values were obtained from all molecularly identified (partial *COI* gene sequences) pheretimoid earthworm species using Tamura-Nei model on software MEGA X 6.0. Results related to intra-species comparison, indicated that lowest p-genetic distance values were observed within member of same species and ranged from 0.00 – 0.004 (for *M. birmanica* specimens) and 0.00 – 0.006 (for *M. posthuma* specimens). Similarly, lower p-distance values were found among member of different species of same genus and ranged between 0.172 – 0.262 (*M. houlleti* and *M. bununa*) or species belonging to different genera 0.220 – 0.550 (*M. posthuma* and *A. minimus*). Therefore, it was concluded that p-distance values comparison within intra and inter species was extremely low and ranged from 0.00 –

0.550 (*i.e.* the difference is <1%). These lowered range values verified the presence of similar traits that were inherited from common origin.

Next, *P. posthuma* coelomic fluid (PCF) was collected through heat and cold shock methods. While, *P. posthuma* body paste (PBP) formation was formed through auto-digestion of earthworm body tissues by self-released coelomic fluid and mucous under sunlight.

Antibiotic sensitivity test was performed to analyze the resistance and sensitivity potential of 5 Gram +ve (*S. aureus* MT448675, *B. cereus* KT182077, *S. pyogenes* CP013840.1, *B. pumilus* JN037409.1 and *P. aeruginosa* MT448672) and 4 Gram -ve (*K. pneumonia* MT448672, *N. gonorrhoeae* AE004969, *E. coli* MT448673 and *P. putida* EU239209.1) bacterial strains against ampicillin (AMP10), doxycycline (DO30), cephalexin (CL30), cloxacillin (OB5), penicillin (P10), oxacillin (OX1), teicoplanin (TEC30) and oxytetracycline (T30) antibiotic disks. Results revealed that maximum zone of inhibition (ZOI) *i.e.* 30.12 ± 0.75 mm was measured for AZM15 against *B. cereus* isolate while minimum ZOI (0 ± 0 mm) was observed for AMP10 (against *B. cereus, P. aeruginosa, P. putida* and *N. gonorrhoeae*), DO30 (against *E. coli*, 0 + 0.25 mm), TEC30 (against *B. cereus,* 0 + 0 mm) and T30 (against *P. aeruginosa,* 0 + 0.5 mm). Moreover, from the results of antibiotic sensitivity test, it was also concluded that all the nine (9) bacterial isolates were sensitive against AZM15 and resistant against AMP10.

To check the antibacterial potential, different concentrations (25, 50, 75 and 100 µg/disc) of PCF and PBP were analyzed by treating Gram +ve (five) and Gram -ve (four) bacterial isolates, along with positive (AZM) and negative (AMP) control. Results revealed that PCF produced ZOIs against both Gram +ve and Gram -ve bacterial isolates ranging from 0.00±0.0 mm (*P. aeruginosa*) to 15.3± 1.0 mm (*B. cereus*) at lowest concentration (25 µg/disc). The ZOIs increased with the PCF concentrations. It was concluded that highest ZOIs were measured as 21.4 ± 1 mm and 25.6 ± 2 mm against *B. cereus* and *B. pumilus* at 100 µg/disc. Similarly, antibacterial effect of PBP was also found dose dependent, as concentration of PBP was increased, the ZOIs were also increased. Because the lowest ZOIs ranged from 4.12 ± 0.0 mm (*S. aureus* and *E. coli*) to 13.11 ± 1.0 mm (*N. gonorrhea*) as measured against all selected

bacteria at 25 µg/disc. While highest ZOIs recorded at 100 µg/disc was 12.2 ± 1.0 mm for *K. pneumonia* and 23.4 ± 2.0 mm for *B. cereus*.

The bacteriostatic (BTS) and bactericidal (BTC) potential of PCF (100 µg/disk) and PBP (100 µg/disk) was analyzed using disk diffusion method against both Gram +ve (five) and Gram –ve (four) bacterial isolates. Results showed that PCF have significant BTS (*i.e.* inhibitory) potential against 4 Gram +ve (*S. aureus*, *P. aeruginosa B. pumilus* and *S. pyogenes*) and 3 Gram -ve bacteria (*K. pneumonia*, *E. coli* and *N. gonorrhoeae*). Meanwhile, PBP have BTS potential against 3 Gram +ve (*S. aureus*, *B. pumilus* and *S. pyogenes*) and 2 Gram -ve (*N. gonorrhoeae* and *K. pneumonia*) bacterial isolates. Like BTS, PCF has also caused BTC (kill) effect against only one Gram -ve (*P. putida*) and Gram +ve (*B. cereus*) bacteria. While, PBP has BTC potential against two Gram -ve (*E. coli* and *P. putida*) and one Gram +ve (*B. cereus*) bacteria. Moreover, the sensitivity profile of both PCF and PBP as antibacterial agents was noticed as 44.44% (*i.e.* 4/9 isolates sensitive to PCF) and 55.56% (*i.e.* 5/9 isolates sensitive to PBP) against selected Gram -ve and Gram +ve bacteria, respectively. Similarly, the cumulative BTS impact of both PCF and PBP was recorded against selected isolates as 66.67% and 55.56%, respectively. Results of BTC showed 33.33% and 44.44% effect of PCF and PBP, respectively against above mentioned isolates.

The biofilm formation time kinetics was examined through crystal violet staining method by utilizing four bacterial isolates (*P. aeruginosa* MT448672, *E. coli* MT448673, *S. aureus* MT448675 and *K. pneumonia* MT448676) as mono-strain culture (MH1, MH2, MH3 and MH4), bi-strain culture (MH6, MH7, MH8, MH9 and MH10) and multiple-strain culture (MH11, MH12, MH13, MH14 and MH15). Results showed that biofilm formation by all strains and their combinations were observed highly significant ($p < 0.005$) between 48 to 72 hours incubation period.

Following time kinetics, the antibiofilm activity of PCF and PBP was examined at different concentrations (*i.e.* 25-200 µg/mL) against 15 experimental combinations (MH1-MH15). Findings of this study revealed that both PBP (5.61±1.0 %) and PCF (5.23±1.5 %) at lowest concentration (25 µg/mL) showed non-significant ($p > 0.05$) antibiofilm activity against all the combinations (MH1-MH15). At 50 µg/mL concentration, both PCF and PBP showed significant ($p < 0.05$) biofilm inhibition (<40

%) against MH1-MH7 and MH11-MH15 combinations, except for MH8 to MH10 combinations (>40 % inhibition). Similarly, both PBP and PCF at 150 and 200 µg/mL concentrations were found highly significant ($p < 0.001$) to inhibit biofilm formation against all strain combinations (MH1 to MH15). More than 90% biofilm inhibition was found against MH1 to MH6 and MH10 combinations at 200 µg/mL of PCF, but it was maximum (96 %) against MH5. In case of PBP, above 96% biofilm reduction (*i.e.* 100 %) was also observed against MH1 to MH10 at 200 µg/mL, while highest inhibition was measured for MH4.

For anti-QS analysis, pyocyanin formation time kinetics was assessed at various incubation periods *i.e.* 0, 24, 48, 72, 96 and 120 hours by using 3 different *P. aeruginosa* strains (*i.e.* PA1 MN900691, PA2 MT448672 and PA3 KT182079). Results showed that PA1 strain synthesized highly significant ($p < 0.0001$) amount of pyocyanin (20 µg/mL) after 96 hours incubation. But, PA2 and PA3 strains significantly formed pyocyanin (18µg/mL) at incubation of 72 hours, respectively. The effective doses (*i.e.* 50-200 µg/mL) of PCF and PBP were further analyzed to eliminate pyocyanin formation, a QS signal synthesized by *P. aeruginosa*. The investigations of this study revealed that 100-150 µg/mL of both PCF and PBP exhibited a marked reduction in pyocyanin formation against three strains (*i.e.* 10 to 5 µg/mL). While, the highest reduction was observed (under 5 µg/mL) at 200 µg/mL against all selected strains. Hence, a concentration dependent increase in QS effect / pyocyanin formation observed in the study.