

Review Article

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Immuno-defense Strategy in Earthworms: A Review Article

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ABSTRACT

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Earthworms are relatively well known group of soil invertebrates and represents significant part of soil biomass. They are routinely exposed with variety of soil microorganisms as well chemical toxicants. Earthworms have developed powerful immunodefense system to potentially escape from pathogenic environment by evoking the immune system and to maintain the self integrity. Earthworm emerges as immuno-defense model because of their strong, well developed innate immuno-defense mechanism strategy against stressed environment. Coelomic cavity of earthworm consists immunocytes/coelomocytes, homologous to vertebrate's leukocytes. Their immunity may potentially involved in detoxification of chemicals including, metal contaminants, pesticides and removal of pathogenic microbes. Their immuno-defense mechanism activates cellular signaling cascade at exposure of contaminants including, nanoparticles by releasing several factor like antimicrobial peptides, PAMPs (pathogen associated molecular pattern), cytotoxic molecules, serine protease pathways. This review aims to understand important cellular and humoral pathways to develop immuno-defense strategy in earthworms that may assist in detoxification of toxicants.

Introduction

Immunity of an organism is a complex system for organism's survivability and integrity. In general, immunity acts like a security barrier for pathogenic environment by defending themselves against invasion of intruders as well as self generated deformed/altered substances. Immune system includes various components like cells, molecules (proteins) and gene that triggers activation of various pathways of the immune response to recognize the non-self molecules.

That act against it to prevent hazardous impact like diseases/ infections by eliminating it from self component. Mechanism of immune responses and their action against pathogen/noxious substances, broadly classified into two categories, innate and adaptive immunity. Innate immunity presents in all organisms as their natural immunity since birth upto the whole life of organisms and is considered as most primitive and simplest form of immunity.

Expression of innate immunity is always “on” but their action start immediately, after exposure of antigen within a short period. This acts like first line of defense for non-specific response against foreign/antigen molecule but it is not able to discriminate the types of antigen because they do not memorize the antigen encounter reaction which has been done previously. After exposure of antigen, innate immunity devour the invaders by means of phagocytosis other associated mechanism of elimination. If it is not happening in that particular regular pathway then they invoke the activation of adaptive immunity just to maintain self integrity and their viability. Adaptive or acquired immunity, are memory based immune responses with high specificity to foreign particles. Antigen encounters with the host and induces the activation of various components. It characterizes the antigen and memorized for the future response to perputate the host integrity. Biological system protects them by destroying and clearing out these digested debris of foreign bodies/particles outside the body. This immune response bears long lasting memory may be life long to act against the same antigen in future security. Annelids are supposed to be the first animal in Phylogenetic tree in which both cellular and humoral immune responses are developed (Dainaut and Scaps, 2001).

Invertebrate’s Immune Responses

During late 0s Nobel Prize winner Elie Metchnikoff described innate immunity mediated by phagocytosis and their importance in eradication of foreign contaminants in Asterias (Beck and Habicht, 1996; Bilej *et al.*, 2010). Soil fertility, regularly checked by their inhabitant faunal diversity and are also indicator for chemical contaminants in soil (Lanno *et al.*, 2004). Directly or indirectly soil resident

invertebrates are exposed to soil for their basic need (Kammenga *et al.*, 2000). Among all invertebrates, soil earthworms are key organism for soil formation and degradation of organic matter in soil. Majority of soil faunal diversity build by earthworms (more than 80%) (Kale, 1997; Nainawat and Nagendra, 2001; Lalthanzara *et al.*, 2011) and are much susceptible to wide range of pathogens that may cause elucidate infection, harmful effect to the host body. Earthworm’s immunity play active role against its foreign pathogenic world and developed immuno-defense strategy. This make them model organism for immunological studies (Cooper and Roch, 2003; Bilej *et al.*, 2010). Majority of study has been done on vertebrate’s immunity but very limited information available (Bilej *et al.*, 2010) in concern with invertebrates including, earthworm’s immunity (Dainaut and Scaps, 2001). During the life cycle of various commercial products, the chemical toxicants like heavy metals, pesticides, hydrocarbons and nanoparticles may be released from these products through normal use that may reaches in wastewater streams that became threat to soil ecosystems. All these anthropogenic activity raises the stress into the environment, may fatal to all tropic level of food chain/web (Kammenga *et al.*, 2000). Therefore, terrestrial eco-systems are expected to be an ultimate sink for a large portion of contaminants. This raises concern about the Immuno-defense strategy in soil organism to encounter these contaminants and earthworms may be potential to organism to bioremediate these contaminants.

Earthworm: Perspective Model for Immuno-Competence

Earthworm’s immunity is most ignorant area of study as compared to other invertebrates

like *Drosophila* and *Caenorhabditis elegans*. In early 1960s first significant study (Cooper and Roch, 2003) were reported in earthworms during the experiment analysis by *allo* and *xeno* graft rejection mechanism based on inflammatory and cellular immune defense responses. This helped to become earthworm as model organism for immune function and their importance in ecological perspectives. As higher organisms, earthworm also possesses both cellular as well as humoral immunity. Earthworm secondary body cavity (coelomic cavity) mesodermal in origin filled with free suspended immuno-protective cells, coelomocytes and also releases various types of humoral factors. This help in earthworm's survivability and integrity against wide range of pathogens, chemical contaminants including pesticides, heavy metals, petroleum hydrocarbon and also nanoparticles of soil ecosystem (Lionetto *et al.*, 2012; Gupta *et al.*, 2014). Coelomic fluid of earthworm potentially controls the pathogens on wide scale (Beschlin *et al.*, 1998; Cooper and Roch, 2003; Bilej *et al.*, 2010) with immuno-defense mechanism through cascade and cellular signaling (Copper *et al.*, 2012) pathways.

Immuno-response in Earthworms

Earthworms belong to the class Oligochaeta of phylum Annelida, are long tubular worm like structure with different variable measurement of size 10 mm to 7 meters long (Julka, 2008). Their Body covered with soft and permeable skin with a layer of cuticle and are metamerically segmented (each segment is separated by septa) with true coelom/body cavity. Coelomic cavity filled with coelomic fluid suspended with coelomocytes and different types of proteins/ peptides. Coelomocytes are the immune cells of earthworm which provide immune defense mechanism against wide

range of foreign bodies. This act as toxicological biomarker which play role in the transportation of toxicants throughout the worm's body (Cooper *et al.*, 2002, Lionetto *et al.*, 2012). Earthworm body lack in skeleton but their coelomic cavity acts like a hydrostatic skeleton which supports its body in various physiological functions like locomotion, movement etc. The flow of coelomic fluid from one segment to another segment is guarded by channels which is present in each segment of body (Cameron, 1932; Bilej *et al.*, 2010). Their body segment is perforated with a pair of nephridia and a pair of dorsal pore that expelled the coelomic fluid at the time of requirement or stress. Body wall of earthworm is slippery moist due to the release of coelomic fluid and mucous from dorsal pore which helps in locomotion, burrowing, entrapping the nutrients/chemicals from soil environment. Their moist body wall rich in coelomocytes, proteins and peptides that lead to immune defense mechanism against foreign bodies, pathogens. Their coelomic fluid may toxic to other vertebrate organism as reported by Salzet *et al.* (2006) in *Eisenia fetida*. Soil pathogen/foreign bodies start invades through their body wall that invoke their immune responses against pathogen.

Cellular Immune Responses in Earthworm

Almost all organisms possess their outer surface coating called skin, that covers the body from the exposure of the outer environment and protect the biological system from microbial/chemical invasion. Epidermis of earthworms acts like a biological barrier for non specific pathogenic foreign bodies. Therefore, their epidermis potentially protects the worm from dermal exposure of pathogens. Skin also secretes polysaccharide mucous/

coelomic fluid, rich in coelomocytes and peptides through nephridial/dorsal pores. These pores are also behaving like a portal of entry for soil bacteria from external soil environment to reach inside the earthworm's body. Microbial invasion may affect the sterility of coelomic fluid. Coelomocytes immediately initiate encapsulation devour invaded engulf pathogens that eliminated out from the body as suggested by Dales and Kalac (1992). Bilej *et al.* (2010) reported phagocytic cells numbers ten times higher than that naturally occurring microbial count total microbial count ($6 \times 10^5/\text{ml}$) in earthworm coelomic fluid.

Calcium Signaling Pathways in Earthworms

Calcium is a primitive molecule, involved in various physiochemical cell signaling pathways *viz.*, gene transcription, muscles contraction and proliferation of cell including their role in immune responses (Petersen *et al.*, 1994; Berridge *et al.*, 1998; Case *et al.*, 2007; Engelmann *et al.*, 2011). Larger proportions of Ca^{2+} occupy endoplasmic reticulum and sarcoplasmic reticulum which reaches to other cell organelles including mitochondria and nucleus (Melendez, 2005); Bootman and Lipp, 2001). They also recorded a small compartment of calcium, as calciosomes in many cell. Pryor *et al.* (2000) stated endosomes and phagosomes also stores calcium in vertebrate leukocytes by activation of calcium signaling. Ca^{2+} also play an important role to induce cell to cell signaling which are related to presence and influx of calcium ion responsible for activation of coelomocytes during exposure of foreign bodies in earthworm (Engelmann *et al.*, 2011). It also act like a secondary messenger (Whitaker, 2006), elevation of intracellular calcium and transient oscillation in the mediator of coelomocytes activation and their metabolism (Engelmann

et al., 2011). However, limited literature is available regarding role of calcium signaling in invertebrates, including earthworms (Burlando *et al.*, 2001; Whitaker, 2006; Engelmann *et al.*, 2011). Several type of influx channel present on plasma membrane for sufficient supply of calcium ion from intercellular space that maintain the cytosolic concentration Ca^{2+} and promote the diverse set of switch "on" and "off" mechanism on plasma membrane (Bootman and Lipp, 2001). Intracellular calcium ion concentration increases due to kinase phosphorylation, activation of transcription factors and expression of selected gene (Oh-hora and Rao, 2008; Engelmann *et al.*, 2011). Molecularly characterized protein calreticulin is a calcium binding evolutionary conserved protein that highly expressed in earthworm's coelomocytes as comparison to their other body tissue (Kauschke *et al.*, 2001). Engelmann *et al.* (2011) assessed subpopulation of coelomocytes, effect or coelomocytes granular, hyaline amoebocytes and chloragocytes for measurement of calcium ion. Characteristic differences were recorded in level of intracellular calcium in effector coelomocytes and in chloragocytes (Opper *et al.*, 2010).

Calcium ion also plays important part in the formation and maturation of phagosome. That initiates the release of Ca^{2+} from endoplasmic reticulum and activates the SOCE channels in the plasma and phagosomal membrane as well. These elevate the oscillatory Ca^{2+} concentration and helps in the ingestion of foreign particles/pathogen. Ca^{2+} is also an important intermediate for solubilization of actin network that covers the phagosome during beginning of phagosome formation and fusion of phagosome with granules containing lytic enzymes (Nunes and Nicolas, 2010).

Immunocytes in Earthworm

Cellular immune response in earthworms depends on activation of various cellular components of their coelom fluid including coelomocytes/immunocytes as well various releasing factors like peptides and proteins. These coelomocytes differentiated on the basis of functional and morphological behavior into three sub population- eleocytes, amoebocytes, and granulocytes. Earthworm's coelomocytes are structurally and functionally similar to vertebrate's leukocytes (Beck and Habicht, 1996; Cooper and Hirabayashi, 2013).

They performed cell-cell adhesion, pattern recognition, phagocytosis, encapsulation, opsonization, lysis, agglutination, inflammation, mitogenesis, rejection and destruction of non-self (allogeneic and xenogeneic) components (Fig.1) from self component with weak memory *in-vivo* and *in-vitro* as well (Cotuk *et al.*, 1984; Cossarizza *et al.*, 1996; Bilej *et al.*, 2010; Cooper and Hirabayashi, 2013). Eleocytes (chloragocytes) are significant immunodefence cell (Lionetto *et al.*, 2012). They remain attached to intestinal lining as chloragogenic tissue and after getting mature, detached from lining. That becomes free-floating in the form of eleocytes (chloragocytes) (Jamieson, 1981; Jamieson, 1992; Affar *et al.*, 1998).

These cells act as a liver in earthworm which are similar to higher organism, helps in detoxification including metal pollution (Cholewa *et al.*, 2002; Kwadrans *et al.*, 2008; Plytycz *et al.*, 2009; Lionetto., 2012). It is supposed chloragogen tissues helps in synthesis of hemoglobulin, to maintain constant cationic concentration and ionic balance in coelomic fluid of earthworm (Prento, 1979, Lionetto *et al.*, 1994; Hatti, 2013). These tissues also play very important role in heavy metal exposure and

detoxification of toxic cation like Zn, Cd etc. (Lionetto *et al.*, 1994; Gupta *et al.*, 2014). They also impart extracellular respiratory pigments and helps in storage of glycogen and lipids (Needham, 1966; Roots and Johnston, 1966; Fischer, 1977; Affar *et al.*, 1998).

Eleocytes produces bactericidal effects or substances that participate in encapsulation and brown body formation of larger foreign pathogen to eliminate them from the body (Cooper and Stein, 1981; Valembois *et al.*, 1992; Valembois *et al.*, 1994; Hatti, 197 2013). Amoebocytes are another type of adherent coelomocytes derived from mesenchymal lining of coelom may be hyaline or granular (Cooper and Stein, 1981). Those helps in wound healing, and also have coagulation property beside transport and storage of nutritive molecules (Adamowicz and Wojtaszek, 2001). Both hyaline and granular amoebocyte have potential to phagocyte foreign bodies followed by pattern recognition multi-phase immune activation that further lead to removal of noxious material from the host body. Cytoplasm of hyaline amoebocytes trap and engulf foreign bodies other lipopolysaccharide, peptidoglycon, beta-glucan etc. Eleocytes and amoebocytes are effector immune defence cell responsible for humoral immunity of worms as well (Cooper and Roch, 1986; Jarosz and Glinski, 1997). Granulocytes, are spherical with centrally located nuclei, acidophilic in nature filled with eosinophilic granules, comprises upto 28% volume of total coelomocytes content (Adamowicz and Wojtaszek, 2001).

Formation of Brown Bodies by Coelomocytes of Earthworms

Coelomocytes recognizes the pattern of pathogen, initially free moving cells gather around the foreign body (like invading

bacteria and particulate wastes), forming the dense capsule, that appears in form of dark brown pigmented aggregates due to presence of melanin pigment, lipofuscin, called as brown bodies (1-2mm in size). This may be due to catabolism of insolubilization of oxidized organic substrates (Cholewa *et al.*, 2006). That process happens with the help of

prophenoloxidase cascade and finally eliminated out from the body (Valembois *et al.*, 1992). Brown body formation usually seen in *Eisenia fetida*, that are enriched in self tissue wastes, agglutinated bacteria and pathogens as well (Valembois *et al.*, 1992), which are further degraded by encapsulation as described in Fig.2.

Figure.1 Expected Phagocytosis Mechanism in Immunocytes/Coelomocytes of earthworm, 1. Oxygen dependent, Oxidative burst initiated by monophosphate shunt, transfer the e⁻ to the NADPH Oxidase. NADPH Oxidase complex present on phagosomal membrane and produce O₂⁻ (superoxide anion) and singlet Oxygen. Conversion of O₂⁻ leads to the production of H₂O₂ (hydrogen peroxide) and then H₂O₂ may generates the activated OH (hydroxyl radical). all together release of these different (OH radical, O₂⁻, singlet Oxygen, H₂O₂) are enough toxic to catalyse the degradation of pathogen/foreign particle potentially. 2. Oxygen independent, fusion of lysosome with phagosome leads to the formation of phago-lysosome, in which lysozymes catalyse the cell wall of foreign particles in low pH. (modified after Morgan and DeCoursey, 2003; Cross and Segal, 2004)

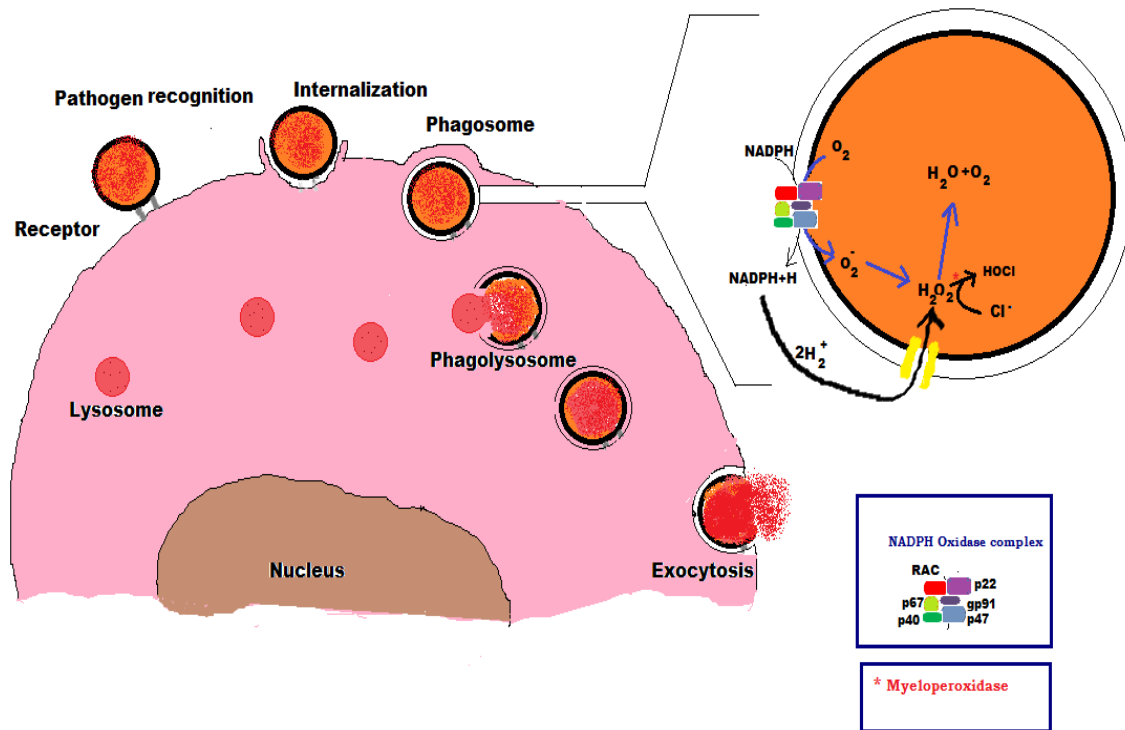
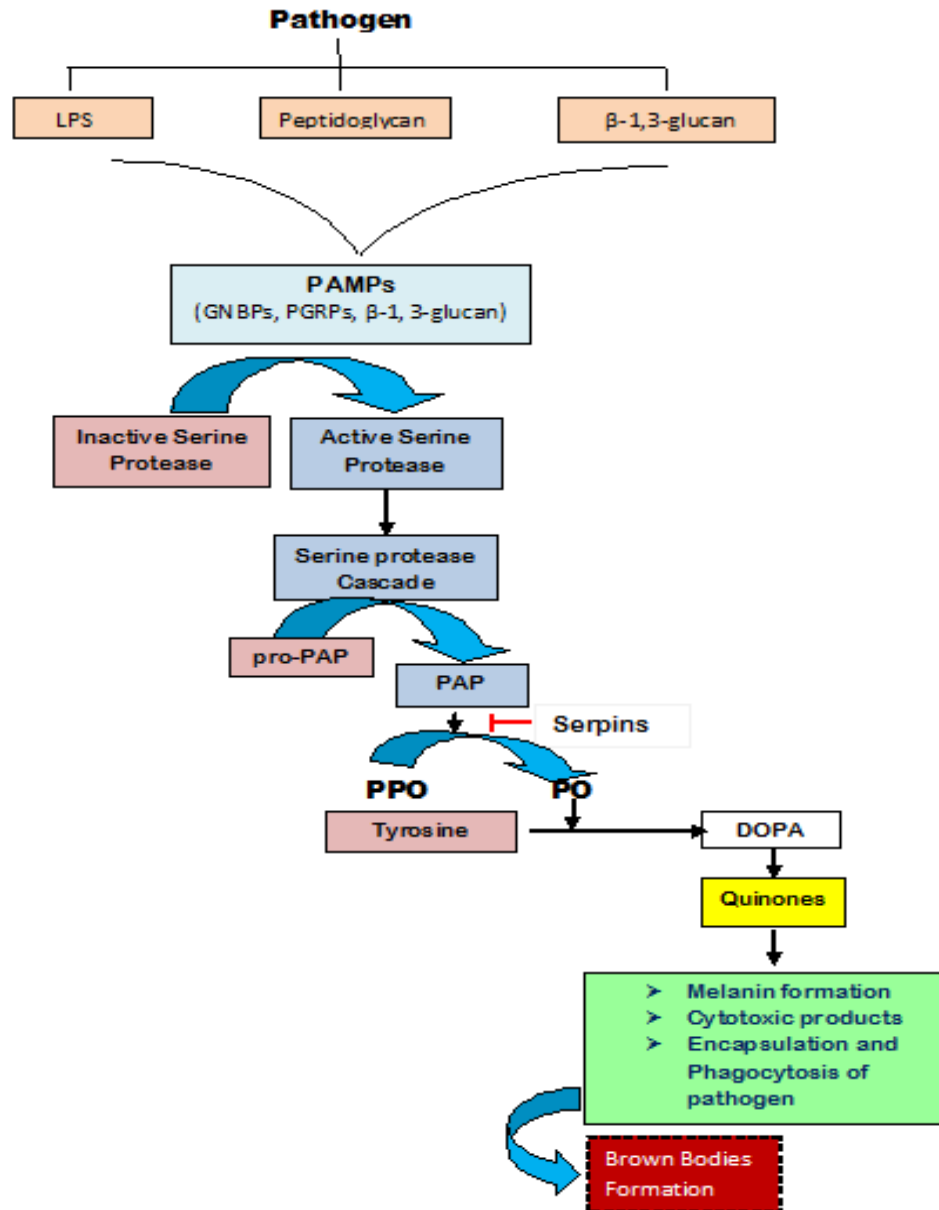


Figure.2 PAMPs (Pathogen Associated Molecular Patterns) are the molecular signature of pathogens like PGRPs (Peptidoglycan Recognition Proteins) Gram +ve bacteria, LPS and GNBPs (Lipopolysaccharide and Gram negative bacteria binding proteins) Gram -ve bacteria, β -1,3-Glucan for fungal pathogen, recognized by immune cells that initiate the activation of serine protease cascade. Serine protease hydrolyzed and activating the prophenoloxidase activating protease (proPAP) to PAP. Serpine are the inhibitor of PAP activation. PPO (prophenoloxidase) hydrolysed by PAP enzymes and converted into activated form PO (phenoloxidase). PO initiate the oxidation of tyrosine to L-3,4-dihydroxyphenylalanine (DOPA) further oxidized to dopachrome and then developed into quinones, which is the precursor of melanin that act release the various antimicrobial factors. (Modified after Beschin et al., 1998; Garcia et al., 2009).



Humoral Immune-defense Mechanism in Earthworms

Coelomic fluid of earthworm is known to act against wide range of pathogen and devour them potentially (Oppen *et al.*, 2012). Quaglino *et al.*, (1996), Joskova *et al.*, (2009) suggested varieties of immunological factors in coelomic fluid of earthworm to maintain their self integrity. These bioactive factors are present in the form of lysozymes, antimicrobial peptides (or proteins) (Cho *et al.*, 1998; Wang *et al.*, 2003; Liu *et al.*, 2004), hemolytic factors like hemagglutination etc. (Bilej *et al.*, 2010). Their fluid also have the potential to digest eukaryotic cell either of same species of different earthworm or other organism like hemocytes of insects and various other derivatives of vertebrates cellular components (Salzet *et al.*, 2006). Their Lysozymes, hydrolytic enzyme shows bactericidal effect against the gram positive peptidoglycan cell wall by catalyzing the intermediate linkage. Thus, they are potentially capable for eradication of gram positive infection in earthworms and may act as sensitive biomarker for Cu ion load and its toxicity in earthworms (Goven *et al.*, 1994).

Role of Antimicrobial Peptides in Immune Responses

Coelomic fluid of earthworm possesses various kinds of antimicrobial peptides of diverse group of different molecular weight. They can enrich the coelomic fluid with strong hemolytic, cytotoxic, hydrolytic, agglutination and opsonization. They are present in different isomeric forms (proline rich antimicrobial peptides Lumbricin I) of antimicrobial peptides in different earthworm sp. Lumbricin I hemolytic activity was first reported in *Lumbricus rubellus* by Cho *et al.* (1998). Isomer of

Lumbricin I, PP-1 (Wang *et al.*, 2003) in *Pheritima tschiliensis* imparts antimucosal defense mechanism in earthworms (Wang *et al.*, 2003).

Recently OEP3 identified in *Eisenia fetida* (Wang *et al.*, 2003) that possess antineoplastic potential and have ability to destroy human cancer cell lines (Cooper *et al.*, 2004). *Eisenia fetida andrei* factor (EFAF) factor contribute efficient proportion in antimicrobial peptides. They have the high ability to lyse the hemoglobin (hemolysis) and other eukaryotic cells. EFAF antimicrobial peptide, either kill the noxious bacteria or arrest the bacterial growth during exposure of pathogen contaminated soil as reported by Du Pasquier and Duprat (1968). That may be bactericidal/ bacteriostatic for pathogenic form of bacteria and protect the worm against wide range of gram positive and gram negative bacterial infection. This hemolytic factor exhibit the agglutination of erythrocytes of blood which may promote the cytotoxic effect (Kauschke and Mohrig, 1987) for foreign bodies That also released by electrocyte in two forms of antimicrobial strong hemolytic peptides namely, fetidin and lysenin. They exhibit antibacterial activity against both Gram-positive and Gram-negative bacteria (Valembois *et al.*, 1982; Lassegues *et al.*, 1989) particularly against strains that are pathogenic for earthworms (Roch *et al.*, 1991; Roch *et al.*, 1987; Valembois *et al.*, 1986). In addition to their bacteriolytic activity, they may also mediate opsonization (Sinkora *et al.*, 1993) and participate in the clotting of the coelomic fluid (Valembois *et al.*, 1988). It was documented, that upon binding to sphingomyelin, a major lipid constituent of plasma membranes of most mammalian cells, polymerize and form 10-nm channels through the lipid bilayer (Roch *et al.*, 1981; Roch *et al.*, 1989). Antimicrobial peptide

(40kD) fetidin, of *E. fetida* have heat labile peptide similar to hemolytic property of vertebrate and other eukaryotic biological system. Ten genetic families of fetidin, characterized with four alleles were reported by Roch *et al.* (1987). Their similarity between these subfamilies depends on presence of aspartic acid, glutamic acid and glycine (Roch *et al.* 1981). This contributes in dermal secretion of mucous, that may checked out the non-pathogenic bacteria population *via* serine protease/serine protease inhibitor pathway (Valembois *et al.*, 1985). During the activation of cell, serine protease release from the cell otherwise it exists as inactive form and remain stored in cell. Another, antimicrobial peptide Lysenin (41 kD) were isolated from *Eisenia fetida* that shares biochemical activities in worm along with two other peptides fetidin and eiseniapore. (Sekizawa *et al.*, 1997). Lysenin exhibits contractile properties as it is experimentally observed in smooth muscle of rat and also impart hemolysis as well because they have high affinity towards sphingomyelin rich cellular membrane such as RBCs, spermatozoa (Cooper and Roch, 2003). Recent study (Bruhn *et al.*, 2006) revealed the few other sub family (multi gene) of lysenin, that has been cloned and known as Lysenin-related protein. Lysenin are highly homologous to fetidin in amino acid sequence (Bilej *et al.*, 2010) and comprises homologous genes. However, variations were recorded in their functional expression in different species of earthworms (Prochazkova *et al.*,2006). The active form of lysenin were also observed in mature free chloragogen tissue in lumen of typhlosole region of intestine (Opper *et al.*, 2012).

Role of Coelomic Cytolytic Factors (CCF)

CCF (42-kDa protein), were observed in soluble form in coelomic fluid of earthworm

Eisenia fetida that performs pattern-recognition molecule activity (Kohlerova *et al.*, 2004). That released by mesenchymal lining of coelomic cavity and free moving coelomocytes.

This helps worm in immunodefense mechanism like cytotoxicity, opsonization and hemolysis of foreign bodies (Beschlin *et al.*, 1998). Molecular characterization and cloning of CCF-1 shows strong homology with the catalytic region of β -1, 3- and β -1, 3-1, 4- glucanases, coagulation factor G from *Limulus polyphemus* and Gram -ve bacteria binding protein in *Bombyx mori* (Beschlin *et al.*,1998). That comprises two distinct domain of lectin, PAMPs (pathogen associated molecular pattern) binding site; there one domain central part of the molecule interacts with O-antigen of lipopolysaccharide (LPS) of gram negative and β -1,3-glucans of yeast and *C-terminal tryptophan* that interacts with peptidoglycan. PAMPs bind specifically invoke CCF to activate the prophenoloxidase (proPO) cascade (Valembois *et al.*, 1986; Salzet *et al.*, 2006).

Conclusions

- 1) Earthworms covers major proportion of soil ecosystem and live together with wide range of beneficial and pathogenic microbiota and also with anthropogenic soil toxicants.
- 2) Earthworms facilitate growth and population of these beneficial microbes to drive coordination of natural scavenging and detoxification of these toxicants.
- 3) Earthworm directly or indirectly exposed to these contaminants and potentially evoked their immune cells activity.

- 4) Their immune system contributes in detoxification of metallic ions toxicity which enters in their body either from ingestion of soil or interstitial pore water of soil in contact with dermal opening of nephridial pore or dorsal pore. A variety of bioactive molecules and peptides present in coelomic fluid of earthworm that helped them to detoxify from soil ecosystem.
- 5) This review is recollection of information regarding earthworms' survivability in toxic/ pathogenic environment and their immuno-defense strategy. The contributed data is in the favor of earthworm as a immuno-defense model of soil ecosystem.

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