The relationship between earthworms and human beings is as old as human civilization. Long before man learned to cultivate crops in the soil, the soil has been inhabited and tilled by earthworms. It is believed that earthworms originated approximately 600 million years ago from aquatic polychaete ancestors. They are considered to be the first multi-cellular invertebrates that have mastered to lead a terrestrial mode of life. However, still they are not completely terrestrial and more appropriately they can be considered to be amphibious.

Attention of man for earthworms has drawn since prehistoric times, mainly because of their extremely beneficial nature and it has been cited in ancient literature throughout the world. They dominate as the major invertebrate fauna in many soils of temperate and tropical regions of the globe. Due to their potential role in soil improvement, attention of philosophers and naturalists was attracted towards them. The importance of earthworms was realized by Greek philosopher, Aristotle (384-322 B.C.) who named them “The Intestine of Earth”.

*Carolus Linnaeus* (1758), White (1789), Savigny (1826), Michaelson (1903, 1910,), Arldt (1908), (Stephenson (1930) were the earlier workers to initiate scientific observations on earthworms. But it was Charles Darwin (1881) who duly recognized the great service rendered by earthworms in decomposition of waste in soil ecosystem. Darwin was supported by a number of contemporary scientists who also believed that earthworms are beneficial creatures to play important part in soil and in maintaining soil fertility (Hensen, 1877; Muller, 1884; Urquhart, 1887; Beddard, 1895; Russel, 1910, Bassalik, 1913).

Since ancient times (< 4000 years, 2600 B.C.), earthworms have been used as food and as medicines for a number of human diseases in various parts of the world including China, Japan, Korea, India, Cambodia, Myanmar (Burma), Vietnam, Iran and Middle East. For external applications, they can be used in healing wounds, chronic boils, piles, sore throat etc. and for internal intake they can be prescribed for chronic cough, diphtheria, jaundice, rheumatic pains, tuberculosis, bronchitis, facial paralysis and impotency.
2.1 Earthworms at world scenario:

In Indian subcontinent various tribal clans use earthworm as food and medicines. Tribal people in Tamilnadu, use earthworm for the treatment of asthma in children below 11 years. For which they take 5 to 6 adult earthworms and dip into 5 g sugar solution and due to ex-osmotic pressure their body fluid oozed out in the form of mucus. This animal syrup is administered to children twice a day for 3 days before each meal. This medicine is reported to offer better relief in asthma and prevents recurrence of the disease in the lifetime of the patients (Solavan et al., 2004). Bala-vaidyas (paediatricians) of peruvannan community of payyannur district of north Kerala use earthworm and other animal products for the preparation of different medicines for treatment of various ailments of children (Unnikrishnan, 2004). The state of Nagaland inhabited by 14 distinct major aboriginal Naga tribe communities and out of them 11 communities consume raw Pheretima species of earthworm as an antidote of snake and spider bites (Jamir and Lal, 2005). Padmanabhan and Sujana (2008) stated that Irular, Mudugar and Kurumbar tribal groups of Attappady hills of Kerela use Pheretima posthuma earthworm for the treatment of healing wounds, chronic boils, piles, sore throat, chronic cough, diphtheria and jaundice in dried form. Oil from this earthworm is used in hemiplegia, paralysis and muscular pain.

In Pakistani traditional medicine system, some animals and their products including earthworm were used for the treatment of different diseases (Ali and Mahdihassan, 2004; Lev, 2003, 2006). According to Vohora and Khan (1978) earthworm have been used in Unani system of medicine to heal wounds, chronic folds, piles and sore throat. Famous Irish scientist of 17th century ‘Robert Boyle’ wrote in his book, “Medical Experiments” that white wine cleansed earthworm convert into powder on moderate heat and then mixed with grains of ambergris (a solid, waxy, flammable substance of a dull grey or blackish colour produced in the digestive system of sperm whales) for both pleasant fragrance and increasing the efficacy of medicine, so that this powder could be prescribed to treat convulsions. Earthworm powder with hens-grease was also recommended as a local application for piles (Fleetwood, 2001).

In the Levant (it is a geographical and cultural region which consist regions of western Asia, eastern Mediterranean and northeast Africa) from the 10th –18th century
whole body of *Lumbricus spp.* of earthworm were used to treat haemorrhoids (these are painful, swollen veins in the lower portion of the rectum or anus), earache, arthritis, obstructions of the urinary tract (Lev, 2003, 2006). Banjo *et al.* (2006) stated that earthworms are used for medicinal purposes, as food and in cultural and religious festivals among Ijebu peoples and natives of Lagos state of Nigeria. Earthworms could be used to treat guinea worm infections in children, barrenness in women and pre and post natal care of pregnant women. These people took earthworms as concoctions (a liquid of different food ingredients) for success in starting new business. They have also used earthworms in rituals as appeasing the gods to bring peace in and around their clan and in traditional ceremonies like weddings and in child naming ceremonies. Lohani (2011) stated that Jirels of Nepal are very much familiar with the medicinal properties of earthworms (*Pheretima spp.* ) and they used them in treatment of different ailments like measles, diarrhoea, jaundice and pneumonia.

In Chinese medical journals, Di Long is a medicinal preparation based on extracts of the earthworm species *Lumbricus rubellus* used in traditional Chinese medicine (TCM) for a wide variety of disorders. It is reported that it can be used to treat convulsions, fevers, rheumatoid arthritis, blood stasis syndromes, chronic bronchitis, bronchial asthma, psychosis, digestive tract ulcer, peptic ulcer, epidemic parotitis, herpes zoster, urticaria, burn, scald, bladder calculi, urinating obstacle, cancer, blepharoptosis or drooping of the upper eye lid along with other phlegm herbs etc. (Liu, 1983; Liang, 1984; Mu, 1988; Wei, 1991; Cheng, *et al.*, 1992; Zhang, *et al.*, 1996; Chen, *et al.*, 1996; Li, *et al.*, 1996; Chen, *et al.*, 1997; Wang, *et al.*, 1998; Zhang, *et al.*, 1998; Liu *et al.*, 2004; Cooper, 2009). Two variants of Di Long use in Chinese culture: (1) Guang Di Long (native to Guangdong, Guangxi and Fujian and collected from spring to autumn) and (2) Tu Di Long (collected during the summer in many regions of China). For initial assays, the abdomen of an earthworm is cut open immediately after capture; then, viscera and other contents are removed. The abdomen is washed clean and dried in the sun or indoors at low temperatures. Di Long has salty and cold properties and is associated with the bladder, liver, lung and spleen meridians (In acupuncture therapy meridians consider as a path through which the life-energy known as “qi” flows). It is thought to work by draining liver heat, by
clearing lung heat and by clearing heat in the collateral channels. Earthworm’s ‘channel opening’ properties are thought to derive from its burrowing habits through the earth. Di Long possess enormous nutritive value and it contain lumbrofebrine, lumbritin, terrestro-lumbrolysin, hypoxanthine, xanthine, adenine, guanine, choline, guanidine, ornithine, lysine, serine, proline, glycine, cystine, valine, phenylalanine, tryptophan, neutral lipids, cholesterol, free fatty acids, triglycerides, complex lipids, phosphatidylcholine, phosphatidyl ethanolamine, phosphatidylserine, dehydrogenase isoenzyme and esterase isoenzyme. Earthworms clearly have offered clues to their value as sources of a healing extract (Cooper and Balamurugan, 2010). The earthworm itself has been used as an anti-inflammatory, analgesic and antipyretic agent in oriental medicine (Nagasawa et al., 1991). In addition, its effects on tumor, epilepsy and blood coagulation have also been suggested by some workers (Wang et al., 1989; Nagasawa et al., 1991; Noda et al., 1992).

2.2 Fibrinolytic/fibrinogenolytic, thrombolytic/ antithrombotic and anti-coagulative activities:

In the end of 19th century Fredericq (1878) and Willem and Minne (1899) observed that an enzyme secreted from the alimentary tract (pharyngeal region, crop, gizzard and anterior portion of the intestine) of earthworm. In 1883, Charles Darwin described that earthworm’s digestive fluids can dissolve fibrin (Zhao et al., 2007). Keilin (1920) isolated a kind of earthworm and found several proteases contained in the worm that could degrade casein, gelatine and albumin. This was the preliminary research about the earthworm proteases. Large-scale research about earthworm proteases began in the decade of 1980. Mihara et al. (1983) isolated a group of proteases with fibrinolytic activity from the earthworm Lumbricus rubellus. However, this group of enzymes is now commonly called as earthworm fibrinolytic enzymes (EFEs) by different research groups (Mihara et al., 1991; Lu et al., 1988 and Zhou et al., 1988). Wu and Fan (1986) were the first who isolate a set of trypsin like proteases from Eisenia fetida, which have different activities like plasminogen activation and fibrin degradation. Different EFEs were isolated and characterized in Amynthas dancatala and Eisenia fetida by Lu et al. (1988) and Zhou et al. (1988) respectively. In a recent inventory of EFEs, many direct acting fibrinolytic/ fibrinogenolytic enzymes were
described, some also with strong anti-oxidative and anti-bacterial activities (Tao et al., 2005).

More proteases have been obtained from different species of earthworm, such as lumbrokinase (Mihara et al., 1991), earthworm-tissue plasminogen activator (Wu and Fan, 1986) earthworm plasminogen activator (Yang and Ru, 1997; Yang et al., 1998 a, b, c), component A of EFE (EFEa) (Tang et al., 2000 and 2002) and biologically active glycolipoprotein complex (G-90) (Hrzenjak et al., 1992 & 1998a, b; Popovic et al., 1998, 2001; Grdisa et al., 2001).

2.2 Different isozymes of earthworm fibrinolytic enzymes:

Several scientific groups have been carried out their research for the invention of new fibrinolytics by using different species of earthworm and employing different purification methods to isolate the enzymes, including ammonium sulphate precipitation, gel filtration, ion exchanging, high-pressure liquid chromatography (HPLC) and affinity chromatography etc. and earthworms have proved themselves for this purpose because earthworm fibrinolytic enzymes are a group of serine proteases, which have strong fibrinolytic and thrombolytic activities (Cong, et al., 2000). Different earthworms exhibit a number of proteolytic enzymes and all those were isozymes but they were named differently in different earthworm species. These isozymes are as follows:

2.2.2 Isozymes from Lumbricus rubellus and their applications:

The earthworm, commonly used for medicine, is Lumbricus rubellus. Mihara et al. (1983, 1990, 1991, 1992) isolated and characterized three fractions F-I, F-II and F-III of fibrinolytic enzymes from the saline extract of earthworm and named lumbrokinase (LK), a collective name for fibrinolytic isozymes proteins. Several investigators purified and further characterized these fibrinolytic enzymes in six isozymes (Park et al., 1989; Mihara et al., 1991, 1992; Nakajima et al., 1993; Cho et al., 2004) and found that they could hydrolyze plasminogen-rich fibrin and plasminogen-free fibrin. F-I and F-III could be subdivided into three F-I-0, F-I-1, and F-I-2 and two F-III-1 and F-III-2 components, respectively, except F-II, which could not be subdivided. The molecular mass of each isozyme had been measured by ionspray mass spectrometry
and they were 23.0, 24.1, 24.2, 24.6, 29.6 and 29.7 kDa. These fibrinolytic enzymes were single peptide chains having more asparagine and aspartic acid residues but less lysine. They had a wide functional pH range (pH 1.0-11.0) and did not inactivate up to 60°C. The highest enzyme activity was exhibited by F-II and F-III-1 around pH 9.0 and at 50°C (Zhou et al., 1997). Fractions-I and III function as chymotrypsin and trypsin like enzyme respectively. Antithrombogenic activity of lumbrokinase, isolated from *L. rubellus*, was enhanced by the immobilization on polyurethane using maleic anhydride methylvinyl ether copolymer (MAMEC) as an enzyme carrier. This immobilized lumbrokinase-polyurethane surface was proved to reduce surface induced thrombus and could become important for clinical application as chemotherapeutic agents (Ryu et al., 1993, 1994, 1995; Park et al., 1999). These enzymes had also showed significant fibrinolytic effect on I¹²⁵-labeled fibrin clots in blood vessels of rabbit pulmonary embolism by oral administration (Shun et al., 1998).

Nakajima et al. (1996, 1999, 2002) had made successful attempt to modify and stabilize earthworm fibrinolytic enzyme F-III-2 from *L. rubellus* by human serum albumin fragment, EDC [(1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide) and PGO (phenylglyoxal). Modification from human serum albumin fragment had masked the undesirable properties of native enzyme like antigenicity, hemorrhagic nature and platelet aggregation. This modified enzyme kept potent proteolytic activity against fibrin and fibrinogen and solubilised thrombus in rat’s vena-cava. Immobilization of F-III-2 isozyme had also been achieved by folded sheet mesoporous material for further stabilization and for continuous fibrinolysis, human serum albumin fragment modified enzyme was immobilized on oxirane-activated acrylic beads without any inactivation of activity at least for one month. Jeon et al. (1995) were purified an anticoagulant/fibrinolytic protease from *Lumbricus rubellus*. The protein was a single chain glycoprotein of 32 kDa that specifically hydrolyzed thrombin and fibrin polymers among the other human plasma protein substrates. According to enzymatic studies this protein was suggested as serine protease having a trypsin like active site and other potential cleavage site which has carbonyl side of arginine. A water extractable anticoagulant had been isolated from *Lumbricus rubellus* by Woo et al. (1996) after complete heat inactivation of endogenous proteases by using different purification methods. Anticoagulant activity in blood was analyzed by measuring
activated partial thromboplastin time (APTT). This protein not only inhibited the conversion of fibrinogen to fibrin but also prolonged the fibrin clot formation. Anticoagulant activity was stable at 100°C for 30 min and in acidic condition (0.4 N HCl). The effect of this partially purified anticoagulant on thrombin were observed with various chromogenic substrates like BAPNA, S-2238, TAME and fibrinogen, only TAME hydrolysis was inhibited among these substrates by L. rubellus anticoagulant protein. In 1998 Park et al., have been successfully purified a fibrinolytic enzyme from Lumbricus rubellus and identified as Lumbrokinase type III which further purified into two sub-fractions Lumbrokinase type III-2 (34.2 kDa) and type III-1(34 kDa). Lumbrokinase type III-1 enzyme exhibited their activity at wide pH range i.e., 2 to 11 and stable up to 65°C. This trypsin like enzyme had higher specificity against fibrin as well as fibrinogen.

In 2000 and 2005 Nakajima et al. had reported that proteases of L. rubellus could be stable at room temperature in buffer solution (Tris-HCl containing sodium azide) for long periods and strongly resistant to organic solvents. Earthworm autolysate was exhibit antioxidant ability and protease activity and its extract could be used as a substitute for cultivation of microorganisms in place of peptone. The serine proteases of L. rubellus also catalyzed the hydrolysis of esters and bio-plastic, especially F-II fibrinolytic enzyme hydrolysed the triacylglycerol.

Mihara et al. (1992) had conducted an experiment on healthy human volunteers to shown the effectiveness of lyophilized powder of L. rubellus as a thrombolytic agent by measuring various parameters in blood like fibrin degradation products (FDP) value, tissue plasminogen activator (t-PA) antigen level and t-PA activities. All the parameters were gradually increased but had decreased and normalized in the end of experiment. These results suggested that earthworm powder could be a possible oral thrombolytic agent so that this powder may thus be applicable for treating patients with thalassemia. Hong et al. (1991); Park et al. (1991); Hahn et al. (1997) demonstrated that intravenous injection of the purified enzyme of L. rubellus showed an antithrombotic effect on thromboembolism in mice and it was also used in the treatment of cerebral apoplexy related to cerebral thrombosis. Oral effectiveness of saline suspension of L. rubellus earthworm powder on rats had also been evaluated by
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Hahn et al. (1997). Several parameters for antithrombotic, anticoagulant and fibrinolytic activities were measured, including platelet aggregation, clotting time, plasmin activity and the levels of FDP (fibrin degradation products), D-dimer, and t-PA antigen. Earthworm powder did not show any effect on platelet aggregation but anticoagulant activity (APTT and TT) was gradually increased and back to normal. Fibrinolytic activity of euglobulin fraction was also high during administration of earthworm powder. The level of FDP was elevated to be comparable to the positive control. They had also stated that oral administration of the earthworm powder was helpful in reduction of venous thrombus induced with viper venom. Complete blood count (CBC) profiles were also within normal ranges. According to these assumptions, they have made in their study, they suggested that the earthworm powder may be valuable for the prevention and/or treatment of thrombotic diseases.

2.2.3 Isozymes from Eisenia fetida and their applications:

Wu and Fan (1986); Zhou et al. (1988) separated at least seven components with fibrinolytic activity from earthworm E. fetida. Of these, three components were separated by a series of purification methods with apparent molecular masses 30, 25 and 30 kDa respectively. They are stable at pH 5.0-9.0 and denatured below pH 2.6. After that, a dimer (45 kDa) of earthworm-plasminogen activator (e-PA) was separated from E. fetida and was considered as a serine protease. Both constituting subunits (MW 26 and 18 kDa) hold together by hydrophobic interaction. The amino acid composition results showed that the small subunit is devoid of ‘Cys’ residue and the large subunit from ‘Lys’. This enzyme (MW 45 kDa) alone and its two subunits (MW 26 and 18 kDa) exhibit different fibrinolytic activities (Yang and Ru, 1997; Yang et al., 1998). Zhao and Jing (2001) observed four glycosylated components, which function like EFE, with molecular masses from 22 to 34 kDa. Li et al. (2003) had isolated a glycosylated component from homogenate of earthworm (Eisenia fetida) with a molecular weight of 34.1 kDa which was different in respect to its N-terminal region with L. rubellus fibrinolytic enzymes. This glycosylated component was considered as a fibrinolytic enzyme because it digests both substrates Chromozym-TH (chromogenic substrate of thrombin) and fibrin specifically. At the same time, Wang et al. (2003) had become successful to purify, characterize and
crystallize seven fibrinolytic enzymes EFE-a, EFE-b, EFE-c, EFE-d, EFE-e, EFE-f, EFE-g from earthworm *E. fetida*. All these proteases showed different fibrinolytic activity on fibrin plates. According to substrate specificity and inhibition studies, they all were belong to trypsin and chymotrysin like enzyme family but ‘EFE-a’ did not show any optimum substrate. N-terminal sequencing indicated their high homology with *L. rubellus* fibrinolytic enzymes. Zhao *et al*. (2006) had found three main components EfP-0-2, EfP-I-1 and EfP-I-2 from *E. fetida* and their thrombolytic activity and toxicity were compared with EFE. They concluded that, among three components, EfP-I-1 had showed higher thrombolytic activity *in vitro* and lower toxicity *in vivo*. Later on, Wu *et al*. (2007) isolated eight fibrinolytic enzymes *Ef* P-0-1, *Ef* P-0-2, *Ef* P-I-1, *Ef* P-I-2, *Ef* P-II-1, *Ef* P-II-2, *Ef* P-III-1, and *Ef* P-III-2. They are all glycoproteins. Two of them *Ef* P-0-2 and *Ef* P-II-2 are new isozymes and the other six in their primary structures are similar to *L. rubellus* fibrinolytic enzymes. Among eight glyco-proteases *Ef* P-1 (M.W. 30kDa), which is located in the epithelial cells of the alimentary canal around the clitellum and has the highest trypsin activity, reacted with its substrates by both the mechanisms, “lock and key” and “induced-fit” of enzyme reactions and these reactions depend on the degree of conformational changes of substrates (Fan *et al*., 2001; Zhao *et al*., 2007; Pan *et al*., 2010). Degradation of chromogenic substrates, plasminogen and fibrinogen was investigated by immobilized EFE-II (isolated as one of the component by Wu and Fan, 1986) and *Ef* P-III-1 (isolated by Wu *et al*., 2007) in two independent research work and the results were concluded that both the enzymes were able to hydrolyze their substrates specifically (Zhao *et al*., 2003; Zhao *et al*., 2007). Pan *et al*. (2011) had discovered a different function of *Eisenia fetida* protease-III-1 (*Ef*P-III-1). They showed that this enzyme not only function as trypsin like protease but also as deoxyribonuclease. Unlike most DNases, this earthworm enzyme recognizes and degrades 5’-phosphate dsDNA in presence of Mg$^{2+}$ instead of 5’OH DNA. This protein acted as a protease under alkaline conditions whereas it exhibited DNase activity under acidic conditions. This dual function of EfP-III-1 may play an important role in the alimentary digestion of the earthworm. A tissue homogenate, named G-90, from *E. fetida* was prepared by Hrzenjak *et al*. (1992). This homogenate was a macromolecular mixture with glycolipoprotein characteristics, which possess numerous biological activities such as
mitogenicity, anticoagulation, fibrinolysis, bacteriostasis, antioxidation and many more in vitro and in vivo conditions. After purification, two fractions P I (34 kDa) and P II (24 kDa) were separated from G-90. Both fractions exhibited the esterase activity, but PI also exhibited amidase activity. A peptidase P I was autocatalytically degraded to P II. Both peptidases and G-90 showed fibrinolytic and anticoagulative activities as well as impact on homeostasis in cardiopathies and malignances of human and dogs (Hrzenjak, et al., 1998 a, b; Popovic, et al., 2001; Grdisa and Hrzenjak, 2007). Different functioning serine proteases EF-SP1 and EF-SP2 were purified and characterized from the coelomic fluid of the earthworm E. fetida (Ueda et al., 2008, 2011). Their MW was 27 and 26 kDa respectively and they were most active at pH 9.5 and 40-60°C. These enzyme exhibited strong antiviral activities against cucumber mosaic virus (CMV) and tomato mosaic virus (TMV). N-terminal amino acid sequence of the enzyme EF-SP1, EF-SP2 showed homology with serine proteases of earthworms, E. foetida and L. rubellus. The enzymatic properties of anti-plant serine proteases (EF-SP1 and EF-SP2) and fibrinolytic enzymes (EFE-d and EFE-e) were similar to each other with respect to substrate specificity, molecular weight and effect of inhibitors.

2.2.4 Isozymes from Lumbricus bimastus and their applications:

This earthworm is one of the important resources for fibrinolytic agents in Southeast-Asian countries, such as Japan, Korea and China etc. (Zhao et al., 2010). Attempts have been made to isolate fibrinolytic enzymes from L. bimastus by some laboratories (Cheng et al., 1996; Xu et al., 2002). Reports have clearly showed presence of three fibrinolytic proteins with apparent molecular masses about 30, 29 and 28 kDa. According to the N-terminal sequences, a c-DNA is obtained by RT-PCR. The c-DNA is inserted in a vector and confirmed by sequencing. The results indicate that the fragment encodes a 242-amino-acid protein called PV242. The whole amino acid sequence is 35% homologous to either F-III-1 or -III-2 of L. rubellus fibrinolytic enzymes. Sun et al. (2002) isolated a lumbrokinase (PI239) from L. bimastus with obvious fibrinolytic activity on fibrin plate. Its c-DNA sequence showed 89.5% homology to those of F-III-1/2 (L. rubellus) and EFE-II (E. fetida). The PI239 protein has the same active and substrate sites as those of t-PA and u-PA.
2.2.5 Isozymes from *Eisenia andrei* and their applications: Lee *et al.* (2007) had isolated a protease fraction SPP-501 from the earthworm *Eisenia andrei*. The antithrombotic activity of isolated fraction was investigated in a thrombosis model of rats and compared to urokinase and t-PA (tissue type-plasminogen activator). SPP-501 reduced euglobulin lysis time (ELT) and did not produce detectable FDPs (Fibrin degradation products). This fraction showed antithrombotic and fibrinolytic activities as well as anticoagulative activity through oral administration. Yan *et al.* (2010) have reported six isozyme fractions F1, F2, F3, F4, F5, F6 from *Eisenia andrei* and their molecular weights ranged from 24.6 to 33 kDa respectively. They did not find out the fibrinolytic activity instead they have showed intestinal absorption of fibrinolytic and thrombolytic fractions of proteases from *Eisenia andrei*.

2.2.6 Isozymes from *Perionyx excavates* and their applications:

Phan *et al.* (2011) have reported six protease fractions, namely FI, FII, FIII-1, FIII-2, FIII-3 and FIV from *Perionyx excavates*. All fractions exhibited strong hydrolytic and proteolytic activity towards casein and fibrin. Fibrinolytic activity of six proteases was ranged from 44 to 831 plasmin unit/mg and ranked as FIII-3 > FIII-2 > FI > FIII-1 > FIV > FII. Degradation of casein was showed at wide range of pH (pH 7-11) and at high temperature (45-60°C) and proteases activities remain unaffected at those variable conditions. They were completely inhibited by phenylmethylsulfonyl fluoride (PMSF). The molecular weights (MW) and isoelectric points (pI) were 27.5-34.5 kDa, and 4.3-5.2, respectively. Amino acid sequence analysis through Mass spectrometry revealed that two peptide FIII-1 and FIII-2 shared 16.9% and 13.2% similarity, respectively, with the fibrinolytic enzymes from two related earthworm species, *Lumbricus rubellus* and *Eisenia fetida*. The *P. excavatus* proteases were classified as serine proteases. They could perform rapid hydrolysis on both coagulated fibrous fibrin and soluble fibrinogen monomers without the presence of activators such as tPA or urokinase. In 2011, Subathra *et al.* has also been investigated fibrinolytic proteases from perionyx excavatus. They have demonstrated six serine proteases isozymes namely PeP-I-0, PeP-I-1, PeP-I-2, PeP-II, PeP-III-1, and PeP-III-2, which were derived from different genes in *Perionyx excavatus*. Among these isozymes PeP-III-1exhibited ability to hydrolyze fibrinogen and to activate plasminogen and
prothrombin, which demonstrated that it is not only take part in fibrinogenolysis but also in fibrogenesis. These results suggested that PeP-III-1 plays a role in the balance between procoagulation and anticoagulation.

2.2.7 Isozymes from Pheretima spp. and their applications:
A novel fibrinolytic enzyme had been isolated from one species of Pheretima by Hu and Fu, (1997). This enzyme was made up of a single chain with M. W. of 22 kDa. It could not only dissolve human thrombi and fibrin directly and strongly but also activate human plasminogen. The enzyme showed little toxic and side effects in animal tests.

2.2.8 Isozymes from Lumbriculus variegatus and their applications:
Tweeten and Reiner (2012) reported several serine proteases, rather than a single enzyme with broad specificity in the tissue extracts of Lumbriculus variegatus. They did not isolate the enzyme instead they treated tissue homogenate of this earthworm with fluorescein-labeled peptide chloromethyl ketone, which specifically binds to trypsin/thrombin-like proteases, which ultimately indicated the presence of proteolytic/fibrinolytic/thrombin activity. On denaturing gel electrophoresis of labelled extract, serine proteases were spotted with molecular weight ranging 28 kDa - 38 kDa.

Although several groups of isozymes have been studied in different species of earthworm but the total number is still not clear. The molecular weights of the proteases of different earthworms are in a relative narrow range (20-35 kDa) and they have activities in a wide pH scope.

2.2.9 Localization of the proteases in an earthworm:
Different group of researchers (Mihara et al., 1991; Zhao et al., 2007) have revealed that earthworm fibrinolytic proteases are expressed and synthesized in the epithelial cells and localized mainly in the crop and gizzard section, particularly in the anterior alimentary regions. In these regions, the proteases maybe contribute to digest protein and peptide in food.
2.2.10 Mechanism of action of Lumbrokinase:

The Lumbrokinase (LK) is a group of proteolytic enzymes, extracted from different species of earthworms, especially from *Lumbricus rubellus*. These enzymes are considered as fibrin specific serine protease and function as plasminogen activator. The mechanism of plasminogen activation by LK is distinct from another thrombolytic enzyme such as urokinase, streptokinase, recombinant tissue-type plasminogen activator, staphylokinase, and recombinant prourokinase (Kasai, 1985; Kim, 1993; Madison, 1995; Verstraete, 2000; Zhao and Li, 2002). Lumbrokinase not only participate in the activation of plasminogen but also activate fibrin directly. These enzymes primarily proteolyses fibrinogen and fibrin and hardly hydrolyze other plasma proteins including plasminogen and albumin (Verma and Pulicherla, 2011). Lumbrokinase is available with different trade names but among them Plasmin Plus capsules was firstly available in China and was approved as a new medicine by Chinese government in 1997 and listed by China National Supervisory & Administrative Commission as "Class 2" nationally protected TCM formula in 1999.

![Diagram of Lumbrokinase mechanism](image)

**Mechanism of action of lumbrokinase on blood (Thrombus) clots. [Adapted by Verma and Pulicherla, 2011]**

In 2000, Chinese govt. included this drug in the National Catalogue of Basic Medicine for the treatment of thrombolytic diseases. Plasmin Plus, which includes
lumbrokinase enzymes as basic ingredient, has three fibrinolytic enzymes: Collagenase, Fibrinolysin (plasmin) & Profibrinolysin activator. After entering in the intestine Plasmin Plus capsule opens up & drug is absorbed in circulatory system. This drug has strong affinity to thrombus & attaches to it. First of all specific collagenase discomposes the collagen shell then fibrinolysin enters the thrombus & dissolves the fibrin and finally profibrinolysin activator also enters the shell & activates more fibrinolysin enzymes. As thrombus disappears, blood vessel is opened & normal blood circulation is restored. Plasmin Plus is nontoxic, good for long term use without any side effects & helps maintain a healthy balance between hemolysis & hemostasis, improves micro circulation, repairs damaged nerve cells & reduce blood sugar. It provides many trace elements and vitamins needed by the human body (Agrawal, 2007).

2.3 Antioxidant, Antiulcer & Hepatoprotective activity:

Saint- Danis et al. (1998) reported the presence of anti-oxidant enzymes, Glutathione, Glutathione related enzymes and catalase in extract of Eisenia fetida andrei. Effect of glycolipoprotein extract (G-90) of Eisenia fetida was investigated by Grdisa et al. (2001) as an antioxidant in cultured human fibroblasts and epithelial cells. G-90 recovered and stimulated cells’ growth after treatment of the cells with H$_2$O$_2$. When the cells were incubated with G-90 before the treatment with H$_2$O$_2$, the oxidative damage of the cells did not occur. These findings suggested that G-90 showed protective effect against the toxicity of H$_2$O$_2$ and stimulated the growth of the cells. Prakash and Ranganathan (2007) had analyzed the antiulceral and antioxidant properties of ‘earthworm paste’ (EPA) derived from Lampito mauritii. Effects of different concentration of EPA were compared with a standard antiulceral drug ranitidine on wistar rats and they found that results of 160 mg/kg EPA on antiulcer and antioxidative markers were considerably effective then other treatments. Hepatoprotective & antioxidant activity of earthworm Lampito mauritii had been studied by Balamurugan (2007) and Balamurugan et al. (2008). They found that whole tissue extract of Lampito mauritii protect the paracetamol induced liver damage in rats by significantly decreasing the hepatic marker enzymes AST, ALT and alkaline phosphatase similar to silymarin in a dose dependant manner. In the same manner,
Prakash et al. (2008) investigated the hepatoprotective and antioxidant properties of indigenous earthworm *Perionyx excavatus* powder using alcohol induced model of hepatotoxic and oxidative damage. Alcohol induced hepatotoxic rats exhibited elevation of lipid-peroxidation and a decrease in the activities of enzymatic and non-enzymatic antioxidant enzymes. Oral administration of dried earthworm powder reversed these parameters towards normalcy. These results suggested that the indigenous earthworm *Perionyx excavatus* could afford a significant hepatoprotective and antioxidant activity.

### 2.4 Anti-inflammatory activity:

Yegnanarayanan et al. (1987, 1988) had evaluated the anti-inflammatory activity of total earthworm paste (TEP) of *Lamptio mauritii* in albino rats. In carrageenan induced oedema model 160mg / kg TEP was found to have the maximum anti-inflammatory activity as noted by percent reduction of paw volume. TEP produces a significant reduction in granuloma weights in cotton pellet induced granuloma pouch method. Further in 1992 Ismail et al. had investigated the anti-inflammatory activity of earthworm based drug “TEP” from *Lamptio Mauritii* and showed that petroleum ether fraction of TEP had ample anti-inflammatory property on albino rats. The efficiency of the drug was appeared to be similar to that of aspirin in carrageenan induced oedema. Anti-inflammatory activity of G- 90 like paste of earthworm *Lamptio Mouritii* was explored by Balamurgan et al. (2009). The results revealed that administration of indomethacin (standard anti-inflammatory drugs) and different doses of earthworm extract (50, 100 & 200mg/kg) restored the normal conditions in a dose dependant manner of histamine & turpentine induced inflammation in rats. In the same manner, Omar et al. (2012) had studied some ethnomedical uses of earthworms. They have studied the anti-inflammatory activity, antipyretic activity and anti-oxidant activity of whole tissue extract of earthworms *Allolobophora caliginosa* Savigny & *Pheretima hawayana* Rosa. They concluded that administration of extracts of both earthworms in a dose of 100mg/kg exhibited better anti-inflammatory activity when compared to standard drug in rats.

### 2.5 Antipyretic activity:

In 1974 Hori et al. reported the antipyretic components in decoctions of Japanese earthworms *Lumbricus spencer, Perichaeta communishima, Goto and Hatai*. They
have stated that these earthworms have been used as an antipyretic drug in folk medicine. *Escherichia coli* were used as pyrogen for inducing fever in male albino rabbits. Results were concluded that antipyretic action of these earthworms decoctions were due to all cis-5, 8, 11, 14-eicosatetraenoic (arachidonic acid) and all cis-5, 8, 11, 14, 17-eicosapentaenoic fatty acids. Balamurgan *et al.* (2009) investigated antipyretic activity of *Lampito Mauritii* and found reduction in hyperpyrexia in rats treated with different doses of earthworm extract and compared with standard antipyretic drug paracetamol. Another study for the evaluation of antipyretic activity of earthworm was conducted by Omar *et al.* (2012) using two earthworms *Pheretima hawayana* Rosa and *Allolobophora caliginosa* Savigny. Pyrexia was induced in rats through *Escherichia coli* and they found that *E. coli* suspension markedly elevated the rectal temperature after administration to rats. Treatments with each earthworm extract (EA and EP) at the doses of 100mg/kg decreased the rectal temperature and were compared to paracetamol.

**2.6 Cytotoxic activity of earthworm:**

*Eisenia foetida* is one of the most intensively investigated species because of the natural occurrence of strong hemolytic activity in the coelomic fluid against erythrocytes of several vertebrates (Du Pasquier and Duprat, 1968). At present much is known about the biochemical properties of this hemolytic compound of the coelomic fluid (Roch, 1979; Roch *et al.*, 1981).

Kauschke & Mohrig (1987) had studied cytotoxic activity in the coelomic fluid of earthworm *Eisenia fetida*. They reported that the *E. fetida* not only causes hemolysis of RBCs of several vertebrate species but also has toxic effect on variety of cell types, such as chicken fibroblasts, guinea pig polymorphonuclear leukocytes and insect hemocytes. However, it has no influence on the vitality of the coelomocytes of *Lumbricus terrestris* and other lumbricides, nor on the hemocytes of the snail *Helix pomatia*, the mussels *Anodonta cygnea* and *Unio tumidus*, free cells of the turbellarian *Euplanaria* sp. or whole *Rhabditis oxycerca* (nematode) and the protozoans *Paramaecium caudatum* and an amoeba of the *Proteus* type.

Further, they observed that cytotoxic effect of CF was seemed to be correlated with the hemolytic activity, since three out of seven haemolytic fractions, which were
separated by electrofocusing, exerted cytotoxic activity. Bilej et al. (1995, 1998) had isolated a cytolytic protein coelomic cytolytic factor-1 (CCF-1) from coelomic fluid of *Eisenia fetida* that displays some functional analogy with mammalian tumor necrosis factor (TNF). This 42kDa protein was responsible for approximately 40% of cytolytic activity of entire coelomic fluid. This CCF-1 protein was involved in cell mediated cytotoxicity because this activity was blocked in the presence of anti-CCF-1 monoclonal antibody. CCF-1 was also involved in opsonising effects and in haemolytic mechanisms that were known to be closely connected with the antibacterial properties of coelomic fluid. Roch et al. (1998) purified and characterized a monomer protein of 14 kDa from *Eisenia fetida*, which was reported as serine protease inhibitor and showed homologies with several plant serine protease inhibitors. They reported that cytotoxic activity of *E. fetida* was due to the presence of two 40 and 45 kDa proteins namely fetidins, which were stimulated by various serine proteases in the coelomic fluid. Hua et al. (2011) isolated a 38.6 kDa protein from *E. fetida* ECFP (earthworm coelomic fluid protein), which activity to chicken possessed significant hemolytic red blood cells.

2.7 Anticancer / Antitumor Activity:

Cancer has been considered as an incurable disease. Although some methods such as surgery, chemotherapy, radiation therapy, and immunotherapy are available, they are far from reaching the goal of complete removal of the cancer cells without damage to the rest of the body. It was demonstrated that the earthworm crude extract had the ability to inhibit the occurrence and development of tumor in vivo (Wang et al., 1986) and to kill the cancer cells directly in vitro (Zhang and Wang, 1987; Zeng et al., 1995). Furthermore, it had been proved that the earthworm proteases enhance the curative effects by both radiation therapy and chemotherapy (Zhang et al., 1991; Zhang et al., 1992). Nagasawa et al. (1991) found a skin extract termed “lombricine” from *Lumbricus terrestris* that could inhibit the growth of mammary tumors in SHN mice. Suzuki and Cooper (1995) observed that coelomocytes of coelomic fluid of *Lumbricus terrestris* may act as effector cells which had potential to destroy tumor cells in vitro. Cytotoxicity of coelomocytes was correlated with effectors and target cell concentration. Results of TEM studies suggested that effector and target cells
should remain in close contact for cytotoxicity. The most malignant tumors secrete urokinase-type plasminogen activator (u-PA). In order to inhibit the hyperactivity of the u-PA, inhibitors of plasminogen activators are synthesized by the surrounding cells for tissue protection, resulting in a high concentration of fibrin locally. The glycolipoprotein mixture (G-90) was isolated from the homogenate of E. fetida (Hrzenjak et al., 1998 a, b; Popovic, 1998; Grdisa, 2001), which was assayed in a euglobulinic test applied to fibrin clot from blood plasma of patients who suffered from malignant tumors. The effect of G-90 on the fibrinolysis rate was related to not only its concentration, but also to histological type where the malignant tumors invade. The blood with the fibrin clots derived from the dogs with cardiopathies and the dogs with malignant tumors was examined for the time of coagulation and fibrinolysis by adding different substances including G-90. The clotting time in the presence of G-90 showed dogs with malignant tumors > healthy dogs > dogs with cardiopathies (Popovic et al., 2001). Recently, a glycosylated component was separated from the earthworm E. fetida by Xie et al. (2003), which had relations with apoptosis of tumor cells. It was highly homologous to F-I-1 and F-I-2 fibrinolytic enzymes of Lumbricus rubellus. It was also identified as a plasmin and plasminogen activator. This enzyme possessed antitumor activity, which was proved by the results of the phase-contrast microscopy observation of apoptotic cells and the localization of fluorescent antibodies in these cells’ nucleus. According to studies of different laboratories, earthworm proteases could be a potential candidate for treating some kind of tumors (Li et al., 2004 and He et al., 2005). Engelmann et al. (2004) analyzed the cytotoxic effects of coelomic fluid coelomocytes on mammalian target cells (HeLa, HEP-2, PC-12 and PA317 cells) and to provide evidence that the lytic factors originate from coelomocytes. The earthworm proteases exhibited obvious anti-tumor activity in the hepatoma cells. The proliferation of the hepatoma cell treated with the proteases was inhibited in proportion to the concentration of the proteases. The growth of tumor xenograft in nude mice was significantly suppressed after being fed with the earthworm protease for four weeks. It had also been found that the earthworm protease can induce apoptosis of hepatoma cells and downregulated the expression of matrix metalloproteinase-2 (Hong et al., 2007). Hua et al. (2011)
reported antitumor effect of ECFP (38.6 kDa protein from *E. fetida*) on HeLa cells and LTEP-A2 cells through MTT assay.

### 2.8 Antimicrobial activity of earthworm:

Earthworms live in an environment filled with various kinds of pathogens. Physiologically and evolutionally, earthworm survive in such an environment, which favour the development of efficient defense mechanisms against various environmental pathogens including the production of certain anti-microbiological substances, especially active proteins and enzymes (Li, *et al*., 2011). Earthworm exhibit not only potent cell-mediated defense reactions but also humoral antibacterial activities (Roch *et al*., 1981; Anderson, 1988) which have been extensively studied in *Eisenia foetida* (Cotuk and Dales, 1984; Roch *et al*., 1986; Stein *et al*., 1986). Coelomic fluid of *E. foetida* was found to have potent antimicrobial activity against six telluric bacteria (Valembois *et al*., 1982) previously shown to be pathogenic for the worms (Lassegues *et al*., 1981). This Annelid coelomic fluid naturally inhibits the in vitro growth of sensitive bacteria belongs to both Gram positive and Gram negative species. Vaillier *et al.* (1985) and Roch *et al.* (1986) had successfully isolated different antibacterial proteins referred to as bacteriostatins (M.W. 45, 40, 20 kDa) from coelomic fluid of earthworm *E. foetida*. A protein lysozyme had also been isolated by Lassalle *et al.* (1988). All these proteins were responsible for the natural antibacterial activity of the coelomic fluid. Lassegues *et al.* (1988) had demonstrated that natural antibacterial activity of earthworm could be enhanced after injecting pathogenic bacteria (*Aeromonas hydrophila* and *Bacillus megaterium*). This enhanced activity did not discriminate between the two pathogenic strains (Lassegues *et al*., 1989). Hirigoyenberry *et al.* (1992) reported that antibacterial activity of *Eisenia fetida andrei* coelomic fluid was mainly mediated by two proteins named 45 kDa and 40 kDa.

Modern medical research has indicated that the coelomic fluid (CF) of earthworms have potent antimicrobial activity against different bacterial strains (Milochau *et al*., 1997; Beschin *et al*., 1998; Wang *et al*., 2003; Liu *et al*., 2004). Cho *et al.* (1998) found a novel antimicrobial peptide, named lumbricin-I from the coelomic fluid of *L. rubellus*, which was purified to homogeneity by a heparin-affinity column and C18
reverse-phase HPLC. Lumbricin-I showed *in vitro* antimicrobial activity against a broad spectrum of microorganisms without hemolytic activity. Different antibacterial peptide were also purified and characterized by some workers like 40 amino acid long ‘antibacterial tetradecapeptide’ from *E. fetida* (Sun, 1997), which could be stored at room temperature for 180 days and PP-1 from *Pheretima tschiliensis* (Wang *et al*., 2003), which showed homology with lumbricin-I from *L. rubellus*. Rivai bakti *et al*., (2003) studied the antimicrobial activity of earthworm extract (*Pontoscolex corethrurus Fr.Mull*) in different solvents such as n-hexane, chloroform and methanol. They reported the antibacterial activity of the extracts on *Staphylococcus aureus* and *Escherichia coli* by recording the inhibition zones. A novel antibacterial short peptide named OEP3121 was purified from the coelomic fluid of *E. fetida*. This peptide was tested against *E. coli, S. aureus* and *Pseudomonas aeruginosa* (Liu *et al*., 2004). Further in 2005 Popovic *et al*., evaluated antimicrobial activity of G-90, a glycolipoprotein mixture of tissue homogenate of earthworm of *Eisenia fetida*. They reported that antimicrobial activity of G-90 may be useful in veterinary & human medicine. In 2007 Ahmad John & Packialakshmi had reported antimicrobial activity of vermiextract of *Perionyx excavatus* against four human pathogenic microorganisms (*E. coli, Proteus, Providencia, Morganella species*). These are well known cause of urinary tract infection in children. They concluded that on continuous taking of earthworm as a meal people can recover from urinary tract infections. Shobha and Kale, (2008) had examined the influence of different preparations from the body of earthworm *Eudrilus eugeniae* on selected soil borne bacterial and fungal pathogens. Body wall and gut extracts were inhibit *Xanthomonas campestris, Ralstonia solanacearum, Erwinia carotovora, Fusarium oxysporum* and *Botryodiplodia theobromae*. Colemic fluid possessed inhibitory effect on *X. campestris and E. carotovora* but any of the extracts didn’t show any activity on *Rhizactonia solani, Alternaria solani* and *Sclerotium rolfsii*. Ansari and Sitaram, (2010) had obtained different results on antimicrobial activity of earthworm. They had reported that different extract of dried earthworm powder showed antifungal activity against *Candida albicans* only but didn’t show any activity on different strains of bacteria like *E. coli, S. aureus, P. aeruginosa*. An earthworm protein (38.6 kDa) from coelomic fluid (ECFP) from *Eisenia fetida* was isolated and purified by Hua *et al*.
(2011) using different isolation procedures. They had tested ECFP on *Escherichia coli* and *Staphylococcus aureus* and found MBC 180μg/ml and 90μg/ml respectively. A different study reported antibacterial activity of *Lampito mauritii* and *Perionyx excavates*. In this study dried powder of these two indigenous earthworms was tested against two gram-positive bacteria *Staphylococcus aureus* and *Staphylococcus epidermidis* and five gram-negative bacteria *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Proteus vulgaris* and *Pseudomonas aeruginosa*. The dried earthworm powder showed strong antibacterial activity against the *S. aureus, P. mirabilis* and *P. aeruginosa*. Powder of *P. excavatus* was found to be more active than the *L. mauritii* (Prakash and Gunsekaran, 2011). Verma and Verma, (2012) isolated, purified and characterized an antimicrobial peptide from ceolomic fluid of Indian earthworm *Pheretima posthumous*. The purified peptide was characterized as serine protease and possessed broad range of antimicrobial activity. The average molecular weight of peptide was found to be 20 kDa and this peptide was also stable towards higher temperature, different pH and various inhibitors. They have evaluated antibacterial activity of purified peptides among physchrophiles, mesophiles and thermophiles.

2.9 Wound healing activity of earthworm:

Being impressed with the versatile potential of earthworm in the treatments of various diseases especially as anti-oxidant and keeping earthworm’s ability to regenerate their amputated parts (Herland and Deligne, 1964; Cooper and Rubilota, 1969; Cooper, 1976; Hrzenjak et al., 1993; Grdisa et al., 2001), a research group had analyzed the efficacy of glycolipoprotein tissue homogenate extract of *Eisenia fetida* (G-90) to activate signal transduction pathways for the production of growth factors *i.e.*, EGF (Epidermal growth factor) and FGF (fibroblast growth factor) in wounds treated with G-90. They measured the activation of EGF and FGF in healthy skin and in wounds with physiological healing also. Results were showed that in the process of wound healing under physiological conditions concentration of EGF increased 2 fold and FGF 1.5 fold but in the case of G-90 treatment both growth factors were increased 10 fold and 5 fold respectively then comparison to healthy skin (Grdisa et al., 2004).
Matausic-pisl et al. (2010) demonstrated the influence of *Eisenia foetida* extract (G-90) on wound healing in an animal model and compared with well established and commonly used wound treating agent ‘Panthenol-D’. They had used excision wound repair model for their study an assessed concentration of micro-organisms on wound as well. They found that the best wound healing effect and shielding from bacterial infections were achieved with G-90 treatment. Besides antibacterial shielding, the wounds treated with G-90 were also protected from inflammation. This extract was shown to shorten the inflammatory and accelerate the proliferative and the maturation phase during wound healing that is why it stimulated the regeneration of an injured epidermis. Thus, they concluded that *Eisenia fetida* earthworm extract (G90) was found to be superior over other treatments and might be considered as a new wound healing agent suitable for use in both veterinary and human medicine practice.

2.10 Role of earthworm in peripheral nerve regeneration and Schwann cell proliferation and migration:

Nerve regeneration is a complex physiological response that takes place after injury like traumatic injury, congenital anomalies, tumor extirpation etc. In mammals, central neurons without myelin sheaths are very difficult to regenerate. However, the peripheral nervous system (PNS) with a myelin sheath exhibits easier regrowth (Bunge, 1993). The regrowth ability results from the intrinsic neuronal activities and surrounding non-neuronal properties in which Schwann cells provide an essential supportive activity for neuron regeneration. Schwann cells differentiate into the myelin sheath of the PNS and can proliferate and migrate into the distal end in the injured nerve area to support axonal regrowth (Fawcett and Keynes, 1990). It has also been reported that Schwann cell migration, which also occurs at the proximal end of the injury area, provides a guide for regenerating axons by interacting with nerve fibers or basal lamina Torigoe et al. (1996). Schwann cell migration is crucial for successful axonal elongation (Anton et al., 1994; Torigoe et al., 1996). Moreover, peripheral nerve injury locally activates Schwann cells and macrophages to synthesize a cocktail of neurotrophic factors, adhesion molecules, cytokines and growth-promoting surface molecules (Ide, 1996; Snider et al., 2002). However, the factors that regulate Schwann cell migration and their signalling mechanisms remain unclear.
Several Chinese medicines have been identified as enhancing neuron regeneration (Tsai et al., 2003). Therefore, neuron regrowth induction using Schwann cells and herbal medicine has good potential for treating injured nerves. The earthworm is a widely used Chinese herbal medicine (Cooper, 2005). A glycolipoprotein mixture of *Eisenia fetida* tissue homogenates G-90 possesses several growth factors and also participates in tissue regeneration and wound healing (Cooper et al., 2004). Wei et al. (2008) had found that a mixed prescription of liquid extract from earthworm showed more obvious improvement in peripheral nerve regeneration than icariin in *in vivo* experiments. Beneficial effect of *Pheretima aspergillum* (E. Perrier) extract on peripheral nerve regeneration was investigated by Wei et al. (2009). For attaining this goal, they had surgically impaired nerve functions by clamping left sciatic nerve and by sham-operation. Results were analyzed by monitoring various parameters like walking track analysis, conduction function of injured sciatic nerve by electrophysiology and regeneration of myelinated nerve by immunohistochemistry. These parameters suggested that earthworm extract appears to enhance sciatic nerve regeneration and function recovery following injury and could be used for the treatment of peripheral nerve injury in humans. Chang et al. (2011a and c) investigated the molecular migration mechanisms in Schwann cells for neuron regeneration induced by earthworm *Pheretima aspergillum* (E. Perrier). Results were demonstrated the roles of MAPK (ERK1/2, JNK and p38) pathways for earthworm-induced matrix-degrading proteolytic enzyme (PAs and MMP2/9) production in Schwann cells. Earthworm induced the phosphorylation of ERK1/2 and p38, but not JNK and activates the downstream signalling expression of PAs and MMPs in a time-dependent manner. Earthworm stimulated ERK1/2 and p38 phosphorylation was attenuated by pre treatment with U0126 and SB203580, resulting in migration and uPA related signal pathway inhibition. The results were confirmed using small interfering ERK1/2 and p38 RNA. These results demonstrated that earthworms can stimulate Schwann cell migration and up-regulate PAs and MMP2/9 expression mediated through the MAPK pathways, ERK1/2 and p38. Collectively results suggested the MAPKs (ERK1/2, p38)-, PAs (uPA, tPA)-, MMP (MMP2, MMP9) signalling pathway of Schwann cells regulated by earthworm might play a major role in Schwann cell migration and nerve regeneration.
Chang et al. (2011b and c) investigated the molecular mechanisms by which *Pheretima aspergillum* (E. Perrier) extract promote neuron regeneration using RSC96 cells. Results showed that treatment with extract of earthworm induces the phosphorylation of the insulin-like growth factor-I (IGF-I) mediated phosphatidylinositol 3-kinase-serine-threonine kinase (PI3K/Akt) pathway, and activates protein expression of cell nuclear antigen (PCNA) in a time-dependent manner. Cell cycle analysis showed that G1 transits into the S phase in 12–16 h, and S transits into the G2 phase 20 h after exposure to earthworm extract. Strong expression of cyclin D1, cyclin E and cyclin A occurs in a time dependent manner. Small interfering RNA (siRNA) mediated knockdown of PI3K significantly reduced PI3K protein expression levels, resulting in Bcl2 survival factor reduction and a marked blockage of G1 to S transition in proliferating cells. These results demonstrated that earthworm promotes the proliferation and survival of RSC96 cells via IGF-I signalling and the mechanism was mainly dependent on the PI3K protein.