DISCUSSION

Earthworms are one of the well known animals on earth, plenty of them emerge from the soil during rainy season and disappear afterwards. They are essential component of soil ecosystem and they affect quality of the soil and in turn get affected by quality of the soil. In general, they are believed to be beneficial creatures as they help in a variety of ways to agriculture. In addition to this aspect, the earthworms have also been consumed as safe and healthy food and they have also been used in prevention and treatment of a myriad of diseases around the world.

Both agricultural and medicinal significance of earthworms were forgotten and neglected in modern human society and in recent past both of these aspects were re-discovered for the safety and sustainability. Inspired from successful discovery of an earthworm-based drug lumbrokinase from *Lumbricus rubellus*, the present study entitled "Evaluation of pharmaceutical significance of the earthworm *Eudrilus eugeniae*" was taken up.

5.1 Anti-oxidant activity:

Different types of toxins, debris and reactive oxygen species, commonly known as free radicals, are generated during metabolism by natural cell death, infections, pollution etc. The side effects of many drugs and medicines are also due to generation and accumulation of such free radicals. They cause many histopathological and physiological disorders leading to degenerative diseases such as ulcers, cancer, hypertension, heart attack, diabetes, malaria, filaria, dengue, hepatitis etc. Their accumulation delays the healing process, hampers normal functions and cause more damage. Thus, these wastes are to be disposed off and removed as early as possible for normal functioning of the body and healing process. In modern medical science it is believed that antioxidants, also known as free radical scavenging agents play an important role. Exogenous intake of antioxidants helps the body to scavenge free radicals effectively and to promote natural functioning of all body systems. (Abd Ghafar *et al*., 2010). Due to this reason, now the doctors are prescribing plant-based antioxidant supplements, in addition to vitamin and mineral supplements. These components are commonly referred as nutraceuticals;

besides free radical scavenging, they also help to reduce the side effects of medicines. Many dieticians recommend antioxidant rich herbs and vegetables for prevention and treatment of diseases. In fact a variety of classes of substances, of natural or synthetic origin including amino acids, proteins, fatty acids, vitamins, minerals, phenolics, phytochemicals etc. act as antioxidants.

Antioxidant and free radical scavenging activity of several foods are due to the presence of many phenolic compounds, mainly flavonoids (Amic *et al.,* 2003). Many flavonoids have antioxidant capacities much stronger than vitamins C and E. A number of reports on plants species have shown positive relationship between total phenols and antioxidant activity (Vinson *et al.,* 1998; Velioglu *et al.,* 1998; Gulcin *et al.,* 2002; Oktay *et al.,* 2003).

Several studies have been conducted to demonstrate potential biological, including antioxidant properties, of bio-therapeutic significance, in crude or refined earthworm extracts. Saint-Danis *et al.* (1998) have reported anti-oxidant enzymes, Glutathione, Glutathione related enzymes and catalase in extract of *Eisenia fetida andrei*. Significant antioxidant activity due to Glutathione (GSH), Glutathione Peroxidase (GPx), Catalase (CAT) and Superoxide Dismutase (SOD) has been demonstrated in earthworm pastes and extracts (Prakash *et al.,* 2007, 2008; Balamurugan *et al.,* 2007, 2008; Chandana *et al.,* 2011; Anitha and Jayrraj, 2012; Omar *et al.,* 2012). Presence of significant amount of polyphenolic compounds, particularly flavonoids has been demonstrated in different species of earthworm tissues and extracts by several workers (Edwards and Bohlen, 1996; Ranganathan, 2006; Balamurugan *et al.,* 2007; Anitha and Jayraaj, 2012, 2013; Verma and Shobha, 2013; Aldarraji *et al.,* 2013). Ranganathan *et al.* (2009) have demonstrated free radicals scavenging activity of *Lampito mauritii* extract against DPPH radicals. Several workers have used different models for demonstration of antioxidant activity in earthworms. Only few of them have used all the models.

In the present study a holistic approach (using *in vitro* and *in vivo* techniques) was used to investigate antioxidant and free radical scavenging activity of earthworm extract. DPPH is a stable free radical which is widely used to test the ability of compounds to act as free

radical scavengers or hydrogen donors, and to evaluate antioxidant activity of different biological extracts and foods. The odd electron in the DPPH free radical gives absorption maximum at 517 nm (purple colour). When this odd electron (free radical) becomes paired with hydrogen from antioxidant (earthworm extract), color of the mixture turns from purple to yellow and optical density is decreased. The decrease in optical density is equivalent to degree of antioxidant activity (Wu *et al.*, 2003).

In the present study, strong *in vitro* anti-oxidant activity of earthworm (*E. eugeniae*) extract was observed in a concentration dependent manner. The minimum (54.13 ± 0.008) antioxidant activity was observed with 1 mg/ml and the maximum (89.24%) was with 4 mg/ml. No further increase was noticed at the concentration of 5 mg/ml, perhaps due to depletion of substrate (DPPH). The DPPH activity of earthworm extract was found to be lower than standard antioxidant Vitamin "C". The findings are in agreement with the results of DPPH assay of Aldarraji *et al.* (2013). They have determined the DPPH antioxidant activity of the extract of two earthworm species and a higher activity (95.23%) was reported for *E. eugeniae* extract than *L. rubellus* (38.26%). Ranganathan *et al.* (2009) and Balamurugan *et al.* (2010) reported 91µg/ml EC50 of DPPH scavenging activity of *L. mauritii*.

The other *in vitro* method of determination of antioxidant activity, used in the present study was reducing power assay in which potassium ferricyanide (Fe^{3+}) is reduced to form potassium ferrocyanide (Fe^{2+}). Minimum (0.260±0.0030) to maximum (0.724±0.0070) reducing power was obtained with 1 mg/ml and 5 mg/ml of earthworm extract with intermediate values at other tested doses (2, 3, 4 mg/ml) respectively. Similar but stronger results of reducing power were obtained for well known standard antioxidant vitamin C. These findings are in accordance with the results of Dahake *et al.* (2010) on reducing power antioxidant activity in the extract of bark of plant *Madhuca longifolia* and Verma and Shobha, (2013) in the dialysed protein extract of earthworm *Pheretima posthuma*, which were less than standard Vitamin "C".

In the present study, total phenolic content of earthworm extract was also measured and high average value (25 µg/ mg or 250 mg /L) was noticed that indicates a high

antioxidant activity. This value was higher than (8.80 µg/ mg) that of Verma and Shobha, (2013) and lower than the values (42.2 µg/ mg) reported by Balamurugan *et al.* (2007) for the species of earthworms *i.e,. Lampito Mauritii*. The results are also in the agreement of the findings of Aldarraji *et al.* (2013). They have quantified high average value of phenolics (220.6 mg/L) in *E. eugeniae* and in *L. rubellus* extract (247.00 mg/L).

High phenolic content has often been considered as marker for high antioxidant activity of the samples that also showed strong inhibition of DPPH radicals and contribute to the high reducing power of *E. eugeniae* extract. The phenolic compounds may contribute directly to the antioxidative action (Duh *et al.*, 1999). It was suggested that polyphenolic compounds may have inhibitory effects on mutagenesis and carcinogenesis in humans, when consumed up to 1.0 g daily from a diet rich in fruits and vegetables (Tanaka *et al.*, 1998). In addition, it was reported that phenolic compounds were associated with antioxidant activity and play an important role in stabilizing lipid peroxidation (Yen *et al.*, 1993).

In addition to demonstrate antioxidant property of earthworm extract under *in vitro* conditions efforts have also been made to demonstrate the influence of earthworm treated male Wistar rats, subjected to acute and chronic anti-inflammatory test, on biochemical markers of antioxidant activity in the blood samples. These parameters included reduced glutathion (GSH), superoxide dismutase (SOD), thiobarbituric acid reactive substance (TBARS) and catalase (CAT). In both the experiments, it was observed that the values of TBARS were significantly lower as compared to the control rats while the levels of other three parameters were significantly higher. More or less similar results were noticed in rats treated with standard anti-inflammatory agent indomethecin. Therefore, it may be concluded that inhibition of inflammation can also be considered to a part of antioxidant activity.

Findings of present study are in the agreement of Balamurugan *et al.* (2007). They have reported anti-inflammatory potential of *Lampito mauritii* in carageenan induced rat paw oedema and turpentine oil induced granuloma pouch model and revealed that the oral administration of the earthworm extract in rats demonstrated a significant increase in the

content of antioxidant enzymes SOD, CAT and GSH which diminished by paracetamol treatment. TBARS concentration was found to be significantly lowered after treatment with earthworm extract, confirming its anti-inflammatory capacity.

A number of workers have demonstrated the presence of Glutathione (GSH), Glutathione related enzymes (Glutathione Peroxidase; GPx), Catalase (CAT) and Superoxide Dismutase (SOD) in earthworm pastes and extracts (Saint- Danis *et al.,* 1998; Prakash *et al.,* 2007, 2008; Balamurugan *et al.,* 2007, 2008; Chandana *et al.,* 2011; Anitha and Jayrraj, 2012; Omar *et al.,* 2012). Though the present experimental setup differs from those of these studies, their findings support similar conclusion that earthworms preparations have antioxidant properties.

The following studies associated with some functional disorder also support the present finding of antioxidant activity of earthworm extract:

- Demonstration of antiulceral and antioxidant properties of 'earthworm paste' (EPA) derived from *Lampito mauritii* in Wistar rats (Prakash *et al.,* 2007).
- Hepatoprotective & antioxidant activity of earthworm *Lampito mauritii* in paracetamol treated rats (Balamurgan *et al.,* 2008).
- Hepatoprotective and antioxidant properties of indigenous earthworm *Perionyx excavatus* powder using alcohol induced rat model of hepatotoxic and oxidative damage (Prakash *et al.,* 2008).
- Effect of glycolipoprotein extract (G-90) of *Eisenia fetida* on cultured human fibroblasts and epithelial cells. The growth of cultured cells was drastically affected by treatment with H_2O_2. It was reported that incubation with G-90 before the treatment with H_2O_2, prevented the oxidative damage of the cells and addition of G-90 in the culture of H_2O_2 cells could recover them from damage and stimulate their growth (Grdisa *et al.,* 2001). These findings not only support the antioxidant property of the G-90 factor, but also suggest that it may help in wound healing.

Almost all health issues involve inflammation, swelling, pain, enhanced cell death, generation of debris, toxins (free radicals), rise in temperature (fever), immune reactions

etc. Exogenous intake of drugs and medicines tend to reverse and optimize the situation towards normalcy. Natural (plant or animal origin) drugs act at various levels and generally show multiple biological activities like anti-inflammatory, antioxidant, immune-stimulatory, anti-pyretic, antimicrobial, wound healing etc. Therefore they are effective in prevention and treatment of a number of diseases. Due to this reason earthworm extracts have shown antiulceral, hepatoprotective, cardio-protective, wound healing properties in experimental animal studies.

5.2 Anti-inflammatory activity:

Inflammation is a primary physiologic defense mechanism of the body that helps to protect it against infection, burn, toxic chemicals, allergens or other noxious stimuli and characterized by pain, redness, swelling, and sometimes loss of sensation. An uncontrolled and persistent inflammation may act as causative factor for many of the chronic illnesses (Sosa *et al.,* 2002). A number of anti-inflammatory synthetic drugs are available, which are effective but may possess several side effects (Bennett and Brown, 2003) and therefore, it is imperative that these synthetic drugs can be replaced with compounds that are equally efficacious, but less toxic and comparatively free from side effects. For achieving this goal researches are going on with full pace around the world to discover newer and less harmful or harmless medicines.

In the present study anti-inflammatory activity of extract of *E. eugeniae* was investigated with the help of two models *i.e.,* acute model (carrageenan induced rat paw oedema) and chronic model (cotton pellet induced granuloma pouch).

Carrageenan induced rat paw oedema model is frequently used as a model of inflammation to determine anti-inflammatory effect of the desired substances. It has also been proved as a useful model in assessing the contribution of mediators involved in vascular changes associated with acute inflammation. Subcutaneous injection of carrageenan into the rat paw produces inflammation resulting from plasma extravasation (Szolcsanyi *et al.,* 1998), increased tissue water and plasma protein exudation along with

neutrophil extravasation. These responses occur due to the metabolism of arachidonic acid via the cyclooxygenase and lipoxygenase enzyme pathways (Gamache *et al.*, 1986).

Oedema formation by carrageenan is a biphasic response. The first phase begins immediately after injection of carrageenan and diminishes in 2 hours and is attributed to the release of histamine, serotonin and bradykinin. The second phase begins at the end of first phase and remains through 3 to 6 hours. The second phase of oedema is correlated with elevated production of prostaglandins, oxygen derive free radicals and production of inducible cyclooxygenase (Panthong *et al.*, 2004). In acute inflammatory response pro-inflammatory agents cannot be eradicated completely that's why chronic inflammation develops which includes a proliferation of fibroblasts and the infiltration of neutrophils and exudation (Arrigoni-Maratellie, 1988; Dunne, 1990). These cells form granuloma, which can be calculated.

The second experiment of the study involved subcutaneous implantation of compressed cotton pellet in rat for induction of granuloma. This model is widely used to evaluate the transudative and proliferative components of the chronic inflammation. The inflammation consists of 3 phases, which are (1) a transudative phase, defined as the increase in the wet weight of the pellet that occurs during the first 3 hours (2) an exudative phase, defined as plasma leaking from the bloodstream around the granuloma that occurs between 3 and 72 hours after the implantation of pellet and (3) a proliferative phase, measured as the increase in the dry weight of the granuloma that occurs between 3 and 6 days after the implantation (Swingle and Shideman, 1972).

Anti-inflammatory drugs like NSAIDs (non steroidal anti-inflammatory drugs) decrease the size of granuloma which results from cellular reaction by suppressing granulocyte infiltration, forbidding generation of collagen fibers and inhibiting mucopolysaccharides (Della Loggia *et al.*, 1968; Alcaraz and Jimenez, 1988). Steroidal anti-inflammatory agents strongly inhibit both transudative and proliferative phases whereas NSAIDs exert only slight inhibition (Swingle and Shideman, 1972). After 6 to 8 days many cells and undifferentiated connective tissue can be observed beside the fluid infiltration which in turn can be measured by weighing the dried pellets after removal. More intensive

granuloma formation has been observed if the cotton pellets have been impregnated with carrageenan (Goldstein *et al.*, 1976).

Results of the present study revealed that treatment of rats with earthworm caused anti-inflammatory response in both groups of rats (carrageenan induced rat paw oedema and cotton pellet induced granuloma pouch) in dose (150, 200, 250 mg/kg) dependent manner and the findings were comparable with the results of indomethacin (standard anti-inflammatory agent) treated rats. The level of carrageenan induced paw oedema was significantly lower than those of the control groups. Treatment of rats with 150 mg EW extract provided lower level of anti-inflammation as paw oedema could not decline to normal during 6 hour regimen. Oedema volume increased from basal volume (1.167±0.0016 ml) to 1.517±0.08 ml at 3rd hour and then decreased to 1.272±0.07 ml at 6th hour. As compared to control values, experimental values (referred as per cent inhibition of oedema) were 40.81%, 44.26%, 50.7%, 68.81%, 71.23% and 82.81% lower for 1 to 6th hour respectively.

Treatment of rats with 200 mg EW extract brought paw volume to near normal level in animals. Here again oedema volume first showed an increase from 1.176±0.0014 ml to 1.516±0.06 ml at 3rd hour followed by a decline to 1.234±0.03 ml at 6th hour. Per cent inhibition values were 44.9%, 47.54%, 52.11%, 69.89%, 75.34% and 92.19% for 1 to 6th hour respectively.

The best results were shown by rats treated with 250 mg EW extract and indomethecin because paw oedema disappeared completely during experimental time of 6 hours as indicated by values of normal paw volume. From these findings, it can assumed that the inhibitory effect of the extract of earthworm *E. eugeniae* on carrageenan-induced inflammation could be due to the inhibition of the enzyme cyclooxygenase, leading to the inhibition of prostaglandin synthesis.

The rats treated different doses of EW extract before implantation of the cotton pellet (chronic test) also exhibited statistically significant reduction in granuloma formation in dose dependent manner. Weight of dry cotton pellet granuloma for 150 mg, 200 mg and

250 mg EW extract treated groups were 62.5±5.49, 54.5±2.72 and 51±4.12 mg and their per cent inhibition of granuloma formation were 40.47%, 48.09% and 51.43% respectively. However the anti-inflammatory activity of earthworm extract was much lower than that of the standard drug indomethecin which showed granulomar weight of 31.3±3.1 mg and per cent inhibition of 70.19%.

The present results dealing with anti-inflammatory activity are in accordance with results of a series of experiments conducted on the influence of petroleum ether extract of earthworm *Lampito mauritii* on anti-inflammatory activity which was maximum at 160 mg/Kg dose (Yegnanarayan *et al.,* 1987, 1988; Ismail *et al.,* 1992). Balamurugan *et al.* (2009) noticed that administration of earthworm extract of *Lampito mauritii* in various concentrations (50-200 mg/Kg) reduced the inflammation in histamine induced rat paw oedema and turpentine oil induced granuloma pouch models.

These results suggested that earthworm extract exerts its anti-inflammatory effect by decreasing granuloma formation because it is assumed that it decrease the number of fibroblasts and demote synthesis of collagen and mucopolysaccharides and in this way it can suppress the proliferative phase of granuloma formation.

Tumor necrosis factor (TNF-α) is a cytokine involved in systemic inflammation which mediate different functions like stimulation of acute phase reaction, fever induction, death of apoptotic cells, inflammation, inhibition of tumorigenesis and viral replication etc. TNF is produced by a variety of cell types including lymphoid cells, mast cells, endothelial cells, cardiac myocytes, adipose tissue, fibroblasts and neurons. Large amounts of TNF are released in response to causative agents of inflammatory agents such as lipopolysaccharide, carrageenan, bacterial products and Interleukin-1 etc. IL-10 is one of the cytokines concentration of which is increased during suppression of inflammation because it down regulates the inflammatory reactions. Studies have shown that IL-10 acts as a potent macrophage deactivator, which blocks TNF-α and other pro-inflammatory cytokines (Trushin *et al.,* 2003). Primarily, it is secreted by activated monocytes, macrophages and Th1 and Th2 cells (Rao, 2005; Mak and Saunders, 2006).

In the present study it was found that treatment of rats with earthworm extract could down-regulate TNF-α and up-regulate IL-10 concentration in both the inflammatory models. Further evidence of dose dependant inhibition of inflammation, as observed by decreasing volume of paw oedema and weight of cotton pellet granuloma, was provided by the determination of values of TNF-α and IL-10. No previous research work has been done to demonstrate the effect of earthworm extract on anti-inflammatory activity in terms of the measurement of TNF-α and IL-10.

From the results of both anti-inflammatory activities including status of anti-oxidant markers and cytokines, it may be concluded that EW extract (250mg/Kg) have significant anti-inflammatory activity because it showed almost similar results as with standard drug indomethacin.

5.3 Anti-pyretic activity:

Fever is an important part of body's defense mechanism against various types of infections, inflammations, malignancy, graft rejection and different diseases. It is characterized by elevated body temperature. In diseased condition fever is good for anybody's body because it can be considered as a battle against infections for the body but in some conditions high temperature may cause harm to brain and if untreated it may leads to death. Zeil and Krupp (1974) stated that fever is the body's natural defence because it creates an environment, which is not suitable for infectious agents and damaged tissue. Hypothalamus regulates body temperature by maintaining balance between production (vasocontriction) and loss of heat (sweating) (Goodman and Gilman, 1996). A number of chemical based medicine are available to suppress fever but long term uses of them causes harm to liver and other body parts such as paracetamol (causes liver damage) so that research is going on to find out newer drugs to conquer high fever, which are effective and harmless to the body.

Anti-pyretic activity of *Lumbricus* and *Perichaeta* species was demonstrated by Hori *et al.* (1974). Later on decline of body temperature (anti-pyretic activity) in yeast induced fevered rats was reported under the influence of different doses of extract of *Eisenia*

foetida (Balamurugan *et al.,* 2009). More recently Omar *et al.* (2012) have reported that treatment of *Escherichia coli* induced (pyrexia) rats with EW extract of *Pheretima hawayana* and *Allolobophora caliginosa* resulted in lowering of the elevated temperature.

In the present study also it was observed that *E. eugeniae* extract was able to reduce the rectal temperature in a dose dependent manner in rats suffering from Brewer's yeast-induced pyrexia. Hori *et al.* (1974) have been shown that anti-pyretic activity of some Japanese earthworm was due to presence of unsaturated fatty acids mainly arachidonic acid. This present study supports above conclusion because extract of *E. eugeniae* was prepared according to Harjenzak *et al.* (1992). According to them the extract of *E. foetida* (G-90) is a glycolipoprotein mixture. Hence it may be possible that extract of *E. eugeniae* was also glycolipoprotein mixture and have fatty acids (arachidonic acid) in it.

5.4 Antibacterial activity:

Increasing burden of antimicrobial resistance in clinically relevant bacteria is an emerging threat around the world. Discovery of new generation antibiotic is challenging task for the researchers as bacteria start resisting soon after introduction of the antibiotics. Furthermore newer drugs are less available and more expensive for resource poor communities. Earthworms live in soil and eat decayed organic materials which have numerous microorganisms; some of them may be fatal to earthworm's lives so that these creatures had developed very efficient defense mechanisms against invading microorganisms to survive themselves in such an environment. Earthworms possess humoral as well as cellular immune system against these microorganisms. Defense system of earthworm is believed to present in their coelomic fluid (Stein *et al*., 1982; Valembois *et al.,* 1982). Different proteins and peptides have been discovered in different species of earthworm which are responsible for their antibacterial activity.

Earthworms kill microorganism by recognizing conserved molecular patterns (lipopolysaccharides (LPS) or peptidoglycans from bacterial cell walls, β-1, 3-glucan of fungal cell walls, and double stranded RNA of viruses) on pathogen's body surface. Recognition of molecules for foreign material has been named as pattern-recognition

proteins (PRPs) (Medzhitov and Janeway, 1997) because the host's primitive effecter cells would recognize molecular patterns rather than particular structures of the invading microorganisms. When bacteria invade into the coelomic cavity of earthworm, the coelomocytes starts to connect with each other by their adhesive structures around the bacteria and form "brown bodies" (Valembois *et al.,* 1992; Cooper *et al.,* 1999). At the same time the coelomocytes intensively synthesize and secrete proteins that adhere to the bacteria, forming aggregations and may inhibit their further proliferation. These proteins attach to the lectin like monosaccharide of the cellular membrane of the bacteria. Different proteins and peptides of different species of earthworms have been extensively studied and different mechanisms of actions have been proposed. Hence, it is very difficult to define which molecule and mechanism of coelomic fluid or extract of earthworms is responsible for its antibacterial activity.

According to Senapathi (1993) earthworm, *Perionyx excavates* contains amazing antimicrobial activity due to their high protein, nitrogen and fat content. Thus extensive research has been done on antimicrobial activity of earthworms described by many authors (Edwards and Bohlen, 1996; Popovic *et al.,* 2005; Balamurugan *et al*., 2007; John and Packialakshmi, 2007; Shobha and Kale, 2008; Ansari and Sitaram, 2011; Hua *et al*., 2011; Prakash and Gunasekaran, 2011; Verma and Verma, 2012; Vasanthi *et al.* 2013; Bhorgin and Uma, 2014).

In the present study, antimicrobial activity of earthworm extract was investigated using some species of fungi and bacteria. No antifungal activity could be demonstrated, but significant antibacterial activity to certain bacteria was noticed. While the other bacterial remained un-affected.

Out of eight bacterial isolates only four of them, gram positive *Staphylococcus aureus* and *Staphylococcus epidermidis,* gram negative *Proteus mirabilis* and *Pseudomonas aeruginosa* were suppressed by earthworm extract. No inhibitory effect was observed on remaining four strain of bacteria *viz. Streptococcus pyrogenes*, *Enterococcus faecalis*, *Enterobacter aerogenes* and *Escherichia coli*. Here the explanation given by Valembois

et al. (1982) may be valid; earthworms have evolved specific antibacterial activity against the bacteria that are potential threat to them.

In the present study earthworm *Eudrilus eugeniae* extract showed a strong significant antimicrobial activity. The results revealed that extract of *E. eugeniae* showed maximum antibacterial activity with gram negative *Pseudomonas aeruginosa.* It showed 29±1 mm diameter of zone of inhibition, which was almost similar (29.5±0.5 mm) to the standard antibiotic streptomycin while minimum antibacterial activity of extract of *E. eugeniae* was obtained with gram positive, *Staphylococcus aureus* with the inhibiotion zone of 21.5±1.5 mm. Results of present study are in agreement of the findings of Prakash and Gunasekaran, (2011). They found that the earthworm powder of two species (*Lampito maurtii* and *Perionyx excavatus*) showed a strong antibacterial activity against the *S. aureus, P. mirabilis,* and *P. aeruginosa.* Findings of this study are also evidenced by Hatti (2014), he has reported that the coelomic fluid of earthworm, *Polypheretima elongata* exhibited highest antibacterial activity against *Staphylococcus aureus.* Khomyakov *et al.* (2007) suggested that antimicrobial agents of earthworms digestive fluid are formed in the earthworm body but not by the soil microorganisms entering their digestive tract.

The extract of *E. eugeniae* is showing highest antibacterial activity against *P. aeruginosa* and failed to show antibacterial activity with *E. coli*. Results of present study are in agreement of Lassegues *et al.* (1989) who had observed that coelomic fluid of *E. foetida andrei* were failed to show antibacterial activity against *Acinetobacter sp.* and *E. coli*. Wang *et al.*, (2007) reported a group of short peptides (AVPF) from *E. foetida* which were failed to inhibit growth of *E. coli* and *Candida albicans* and showed maximum activity against *P. aeruginosa*. Same results were found with this *E. eugeniae* extract study. Shobha and Kale (2008) suggested that the gut extracts of *E. eugeniae* have antifungal activity but the extract of *E. eugeniae* used in the present study didn't show any antifungal activities. Results of present study were also supported by Vasanthi *et al.* (2013) and Anitha and Jayraaj (2013) who have worked on *E.* eugeniae but they are different only on results of *E. coli* and *C. albicans*. Prakash (2013) reported that

earthworm powder was used against various ailments in indigenous system of medicine which was found to be fruitful against microorganisms.

According to the results, it can be concluded that extract of *Eudrilus eugeniae* can be used to formulate a new natural antimicrobial product for controlling infection of multidrug resistant bacteria where treatment is very difficult as the drug of choice for treating infection doesn't work.

5.5 Wound healing activity:

Wounds are inescapable events in life and they may arise due to physical, chemical or microbial agents (Harshmohan, 2005). The first and most significant protective barrier against these damaging factors is skin and creation of a wound results in an opening or breaking of the skin and it permits more easy entry of the damaging factors. Healing of the wounds is an intricate process which involves a chain of well organizes biochemical and cellular events leading to the growth and repair of wounded tissue (Gailit and Clark, 1994). Wound healing can be broadly categorized into three overlapping stages: (1) inflammatory phase, which establishes hemostasis and inflammation (2) proliferative phase, which involves granulation, contraction and epithelialization (3) remodelling phase, which determines the strength and appearance of the healed tissue (Evans, 1980).

When skin tissue is damaged (wounded), blood comes in contact with collagen which triggers platelets aggregation and clot formation to prevent blood loss and obtain hemostasis. Inflammatory and growth factors are released from the aggregated platelets. During inflammatory phase various chemokines and cytokines are released and they attract phagocytic cells to consume cell debris, bacteria and damaged tissue. As inflammatory phase declines some signaling molecules are released that initiate the proliferative phase of wound healing.

Inflammation is also associated with swelling or oedema due to accumulation of fluids in the wound area and pain due to sensitization of nerves.

The proliferative phase includes angiogenesis, collagen deposition, granular tissue formation, epithelialization and wound contraction. New blood vessels are formed

by vascular endothelial cells through angiogenesis. Granular tissue is formed by the growth of fibroblasts which in turn form a new provisional extracellular matrix (ECM) by excreting collagen and fibronectin. Subsequently re-epithelialization occurs to provide a cover for the newly formed tissue on wound bed through the proliferation of epithelial cells. After that wound becomes smaller or contract by the action of myofibroblasts which arise from the stimulation of fibroblast by the action of growth factors. In third (last) maturation and remodeling phase, collagen is remodeled and realigned along tension lines. The cells that are no longer needed are removed by apoptosis and phagocytosis.

Modern medical science (Allopathy) is recording new achievements day by day and newer drugs are coming up for the treatment of different diseases. Most of the medicines are based on synthetic chemical agents and majority of them have negative side effects (Weinstein-Oppenheimer *et al.,* 2010; Sasidharan *et al.,* 2010; Khalid *et al.,* 2011; Ahmad *et al.*, 2011). According to western pharmacopoeia only 1-3% drugs are used for skin diseases and wound healing while one third of herbal remedies are used for such diseases (Balick and Cox, 1996). The value of traditional medical practices as affordable healthcare has also been recognized by World Health Organization (WHO) (Olajuyigbe and Afolayan, 2011).

Earthworms have been used for treatment of different diseases form thousands of years in many parts of the world and its traditional medical knowledge has been proved scientifically by different scientific groups. In the present study, results of investigations on a number of biological activities (anti-oxiodative, anti-inflammatory, anti-pyretic, anti-microbial and fibrinolytic activities) of earthworm have been described, which could be helpful in development of earthworm based drugs. Looking to the significant antioxidant, anti-inflammatory and anti-microbial activities of earthworm extract it was decided to study wound healing activity of earthworm extract by topical application of ointment of test material on excised wound model (Chithra *et al.,* 1988; Babu *et al.,* 2003) of experimental rats. Observations were recorded on wound contraction percentage, epithelialisation time and histological changes during healing process.

The rats treated with Betadine (positive control as standard healing drug) showed satisfactory healing influence with 97.95±0.30% wound contraction percentage and epithelialisation time of 16.80±0.21 days. The wounded rats treated with EW extract ointments showed progressive healing process and even better results than Betadine were obtained. Rats treated with 50 mg earthworm extract ointment showed 100% wound contraction percentage and epithelialisation time of 14.17±0.30 days. Results of lower (25 mg) and higher (100 mg) doses of earthworm extracts showed comparatively lower influence on these parameters.

In the present study higher percentage of wound contraction percentage and lower time of epithelialisation were indications of better healing. Histological observations also provided consistent results of healing parameters showing varying status of regenerated squmaous epithelium, deposition of collagen fibers, lesser number of lymphocytes, copious amount of tiny blood vessels and enormous and well formed sweat glands and hair follicles. In other groups of rats varying degrees of healing conditions were noticed.

Grdisa *et al.* (2004) found an increased concentration of epidermal growth factor (EGF) and fibroblast growth factor (FGF) at 6 h after wounding. In comparison with healthy skin, the concentration of EGF increased 10-fold and FGF five-fold in wounds treated with G-90 (EW extract of *E. foetida*). Similar results were reported by Matausic-Pisl *et al.* (2010) in their study on wound healing properties of extract of *Eisenia foetida*. From these findings, it appears that treatment with earthworms extract promote synthesis of growth factors which in turn enhance the proliferation of epithelial cells and collagen formation. Collagen is a family of proteins which provide structural support to wound on which various cells are formed and migrate to close the wound. The results of present study were consistent with these studies.

It was also observed in the present study that EW extract possesses significant amounts of antioxidants and phenolic compounds, which are known to reduce lipid peroxidation not only by preventing or slowing the onset of cell necrosis but also by improving vascularity and regenerative events. Hence, any drug that inhibits lipid peroxidation is believed to increase the viability of collagen fibrils by increasing the strength of collagen fibres,

increasing the circulation, preventing the cell damage and by promoting the DNA synthesis (Getie *et al.*, 2002). Phenolic compounds (Scortichini and Pia Rossi, 1991; Tsuchiya *et al.*, 1996) of plants are also known to promote the wound-healing process mainly due to their astringent and antimicrobial property, which seems to be responsible for wound contraction and increased rate of epithelialisation. EW extract possess high amount of phenolic compounds which is also a possible cause of wound healing activity of it. One more possible reason behind wound healing activity of EW extract may be the presence of anti-inflammatory activity in EW extract. Newton *et al.* (2004) stated that presence of high numbers of macrophages in inflammatory phase of wound healing actually delays wound contraction and thus the disappearance of macrophages from the wound may be essential for subsequent phases to occur. So that it may be concluded that anti-inflammatory impact of EW extract reduce the numbers of macrophages in wound and in this way fastens the wound healing by the proliferation of fibroblast which in turn secrete growth factors that attract epithelial cells to the wound site because these cells are responsible for epithelialisation. Along with the fibroblast proliferation, neovascularization (angiogenesis: formation of new blood vessels) also occurs after the introduction of endothelial cells at wound site because newly formed blood vessels provide oxygen and nutrients to fibroblast and epithelial cells to grow. Numerous blood vessels were seen in the histological analysis of the healed skin of wound of 50 mg EW extract ointment treated rats.

5.6 Fibrinolytic, Proteolytic, Fibrinogenolytic Activity of Earthworm Extract:

Human body is complex structure which is made up of millions of cells organized in the form of organs which function in a coordinated manner under optimal environmental conditions. The lifestyle of human beings is changing in a faster way with modernization with increasing comforts and facilities. The other side of the picture is not so pleasant due to increasing environmental pollution, global climatic changes and newer environment and health hazards. The everyday life of people is becoming hectic, they are distracting from healthy life style and healthy food which are resulting in increasing incidence of lifestyle health problems such as cancer, diabetes, obesity, hypertension, myocardial

infarction, coronary heart disease, atherosclerosis, thrombosis of various parts of the body etc. Cardiovascular diseases are most prevalent around the world and become leading cause of the human death. Among all kinds of cardiovascular diseases, thrombosis is a frequently occurred symptom (Wang *et al.* 2006). For thrombolytic therapy, the main drugs are urokinase (UK), streptokinase (SK) and tissue plasminogen activator (t-PA) but they are too expensive and their half-life is short. These standard fibrinolytic drugs are heat labile and have to be stored under refrigeration and are given through intra venous route as they are not effective through oral route. Besides this, they have the side effect like hemorrhage. Therefore great attention has been directed towards in search of thrombolytic agents of various origins. Different bioactive components from animals and plants can be used as an alternative treatment for a number of cardiovascular diseases.

Earthworm based drugs because of its anticoagulative and fibrinolytic activities (Mihara *et al.,* 1991; Hrzenjak *et al.,* 1992; Nakajima *et al.,* 1993; Zhu *et al.,* 1993; Jeon *et al.,* 1995; Popovic *et al.,* 1996; Yang and Ru, 1997; Hrzenjak *et al.,* 1998 a and b; Popovic *et al.,* 2001; Lee *et al.,* 2007) may be a boon for patients who have different degrees of arterial blockage or have strong tendency of clot formation because unlike standard fibrinolytic drugs, earthworm based drugs can be stored at room temperature as they are heat resistant and work through oral pathway as well. When a trauma (internal or external) occur, a tissue factor thromboplastin released (Guyton and Hall, 1998; Hrzenjak *et al.,* 1998 b; Popovic *et al.,* 2001) which in turn activate different factors of either intrinsic or extrinsic pathway of blood clotting and ultimately form prothrombin activator which then convert prothrombin to thrombin. This thrombin acts as a proteolytic enzyme and split fibrinogen to fibrin monomers. These fibrin monomers have automatic polymerization capabilities with other fibrin monomers and form a weak polymerized fibrin meshwork. Further, this weak interaction of fibrin monomers strengthen by the action of activated fibrin stabilizing factor (XIIIa) and form a strong interactions between fibrin monomers which entrap blood cells, platelets (thrombocytes), plasma. This meshwork is known as clot which ceased the blood flow from traumatized area.

Every new drug, developed for the treatment of blood clot related diseases should be thoroughly tested to prove itself capable for recommending them for treatment of these diseases. Hence, in this present study earthworm extract of *Eudrilus eugeniae* was explored for its anticoagulative and fibrinolytic activity.

The fibrinolytic activity of earthworm extract was analyzed by using plasminogen free fibrin plate method and plasminogen rich fibrin plate method and the results were compared with those standard fibrinolytic drug streptokinase. Streptokinase is a bacterial protein which was isolated through β- hemolytic streptococci and is considered as plasminogen activator (Tillett and Garner, 1933). According to Christensen and MacLeod, (1945) (inventor of streptokinase) and Marder and Sherry, (1988) this protein is not fibrinolytic in itself but interacts with lytic precursor of blood clotting system plasminogen to convert it in a proteolytic enzyme plasmin. Therefore it is used on plasminogen rich plate for comparison of results of fibrinolytic activity of both methods. A strong fibrinolytic activity has been observed in both the methods. Results obtained showed that fibrinolytic activity on plasminogen rich plate was 1.27 times higher than plasminogen free plate. Plasminogen rich plate also showed higher activity than all concentrations of streptokinase. It is 2.4 times higher than the highest concentration of streptokinase (10,000 Units/ml) while plasminogen free plate showed 1.88 times higher activity than the same concentration of streptokinase. Specific activity of plasminogen rich fibrin plate was 1.28 times higher than plasminogen free plate and also showed higher specific activity than comparison to all concentrations of streptokinase. In comparison to the highest streptokinase concentration (10,000 Units/ml), plasminogen rich fibrin plate showed 2.39 and plasminogen free plate showed 1.87 times higher activity. According to the above findings it may be conclude that EW extract of E. *eugeniae* acts not only as fibrinolytic by showing direct digestive action on fibrin clot but also as plasminogen activator as observed on plasminogen rich plate, while standard drug streptokinase showed only plasminogen activator activity but it doen't has direct digestive action on fibrin clot. These findings were supported by a number of studies (Park *et al.,* 1989; Mihara *et al.,* 1989, 1991; Jeon *et al.,* 1995; Yang and Ru, 1997; Wang

et al., 2003; Nakajima *et al.,* 2003; Cho *et al.,* 2004, Subathra *et al.,* 2011; Phan *et al.,* 2011, Trisina *et al.,* 2011) carried out on different species of earthworms.

Euglobulin is a serum globulin protein fraction which is insoluble in water but soluble in saline solution. It consists of both types of proteins which take part in clot formation and in clot dissolution in blood. It can be isolated from blood by acid precipitation and then converting in clot by the addition of $CaCl_2$, because Ca++ is essentially involved in catalyzing various blood clotting steps. Euglobulin fraction (after formation as a clot) dissolves itself automatically because of the presence of physiological tissue type plasminogen activator (t-PA) which converts plasminogen into plasmin which in turn dissolve euglobulin clot. Every species has its normal range of euglobulin clot lysis time (ECLT). In adult human it is 90-320 minutes (Smith *et al.,* 2003). This test is a useful indicator of disturbances in *in-vivo* fibrinolysis in many physiological as well as pathological conditions like acute myocardial infarction, disseminated intravascular coagulation (DIC) etc. (Copley *et al.* 1959; Markarian *et al.* 1967; Burns *et al.* 1984; Urano *et al.* 1990; Reverdiau-Moalic *et al.* 1991; Cheras *et al.* 1997; Borawski and Mysliwiec, 2001). In spite of it, if additional plasminogen activator is added to this euglobulin clot, the lyses time will be shortened. This fact can be used to assess the fibrinolytic activity of new drugs of thrombolytic therapy. Findings of present study shown that EW extract of *E. eugeniae* was able to shorten ECLT by 74.14 % and 45.58 % faster than average normal physiological ECLT (245 minutes) and ECLT of streptokinase respectively while streptokinase shorten ECLT 52.49 % faster than comparison to the average normal physiological ECLT. These results are supported by the findings of Hrzenjak *et al.,* (1998 a, b), Lee *et al.,* (2007). In these results EW extract showed better performance of lyses of euglobulin clot than by standard drug streptokinase and in fibrin plate methods it also showed lyses of fibrin clot on both plasminogen free and plasminogen rich fibrin plates so that it may again be proved the potential of EW extract to act as fibrinolytic as well as plasminogen activator to activate plasminogen into plasmin to lyses blood clots. Kowalski *et al.* (1959) and Borawski and Mysliwiec, (2001) reported that increased concentration of fibrinogen in euglobulin clot significantly

prolongs the ECLT, hence it may be concluded that ECLT results of 9 healthy volunteers vary significantly with each other in both the test (EW extract and streptokinase treatment).

From the above studies the role of EW extract as fibrinolytic agent was proved. But an effort was made to find out whether EW extract of *E. eugeniae* is able to hydrolyze other protein substrates such as casein. It was confirmed that earthworm extract had a good proteolytic activity having a specific protease activity of 18.20±0.50 Units/mg. Findings of this present study are in accordance with the results of Cho *et al.* (2004); Lee *et al.* (2007) and Trisina *et al.* (2011). These scientific groups observed specific activity from 11.3 to 167.5 Units/mg in separated fractions and extracts of earthworms *Lumbricus rubellus* and *Eisenia andrei*. A higher activity was also reported in different earthworm species by other authors (Nakajima *et al.*, 1996, 2000; Yan *et al.*, 2010). Less specific activity was also reported by Phan *et al.* (2011) in the autolysate of *P. excavates* which was 0.138 Unit/mg. Only Park *et al.* (1998) reported contradictory result where they reported lower caseinolytic activity of lumbrokinase III-1 (proteolytic enzyme of *L. rubellus*) than trypsin.

It was reported that earthworm enzymes have the ability to digest fibrinogen and fibrin directly (Mishra and Dash, 1980). This fact was further strengthened by the studies of a number of scientific groups (Jeon *et al.*, 1995; Nakajima *et al.*, 1996; Park *et al.*, 1998; Zhao *et al.*, 2003, 2007; Trisina *et al.*, 2011). In the present study fibrinogenolytic activity of *E. eugeniae* extract was investigated using analysis by SDS-PAGE. The Aα and Bβ chain of fibrinogen cleaved within one minute and γ chain degraded in one hour. These results were found to be better than the findings of Jeon *et al.* (1995) and Nakajima *et al.* (1996). They have investigated their study on the purified enzyme of L. *rubellus* and showed rapid degradation of α, β chains and slower degradation of γ chain. These results confirm that EW extract of E. *eugeniae* has strong α, β-fibrinogenase and moderate γ- fibrinogenase activity. It was also proved that EW extract behave differently from the typical blood clotting enzyme thrombin because it hydrolyzes fibrinogen. This is

the first report which describes fibrinogenolytic activity of *E. eugeniae* and proved higher activity than other species of earthworms.

The anticoagulative activity of EW extract of *E. eugeniae* may be explained by fibrinogenolytic activity (direct hydrolytic action on fibrinogen) and plasmin like activity on fibrin. Further it also possesses a component (plasminogen activator) that converts plasminogen to plasmin first than this plasmin exerts its effect. Bovill *et al.* (1995) and Dempfle *et al.* (2000) described that the decrease of fibrinogen leads to an anticoagulation effect in blood circulation.

The present study experiments to demonstrate biological activities of earthworm, *E. eugeniae* using *in-vitro* and *in-vivo* (male Wistar rats). In some experiments blood of human volunteers was also employed. It is for the first time that such an expensive investigation has been made. In earlier studies different groups of workers have investigate different aspects. In brief the findings of the study revealed that earthworm extract has significant and highly impressive biological activities (anti-oxidative, anti-inflammatory, anti-pyretic, anti-microbial, wound healing, fibrinolytic, proteolytic and fibrinogenolytic activities) of pharmacological significance. It can be recommended that EW extract be taken for further studies with an objective of "new drug discovery" from a natural and renewable resource.