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Therapeutic Properties of Earthworms

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ABSTRACT

The medical value of earthworms has been known for centuries. This is evident from the history of the ancient Southeastern Asian medicine (China, Japan, Vietnam). The earthworms are the source of proteins, peptides, enzymes and physiologically active substances. Thus, the extracts prepared from the earthworm tissue have been used for the treatment of numerous diseases. Earthworms, like other complex invertebrates, possess several types of leukocytes which synthesize and secrete a variety of immunoprotective molecules. The immunoprotective system is involved in phagocytosis, encapsulation, agglutination, opsonization, clotting and lysis of foreign components. The lytic reactions against several targets are mediated by two major leukocytes, small and large coelomocytes. In the last 10 years a number of earthworm's clot-dissolving, lytic and immune-boosting compounds have been isolated and tested in laboratory and clinical studies. In particular, research has been focused on clot-dissolving molecules. Fibrinolytic enzymes, which are regarded as potent and safe, have been purified and studied from several species of earthworms, including *Lumbricus rubellas* and *Eisenia fetida*. Its therapeutic and preventive effects on thrombosis-related disease have been clinically confirmed. However, several studies have shown that earthworm extracts contain different macromolecules, which exhibit a variety of activities, such as antioxidative, antibacterial, antiinflammatory, antitumor, etc. Some of these activities are involved in wound healing using an earthworm preparation.

Keywords: diseases, fibrin clot, immune system

Abbreviations: CCF-1, coelomic cytolytic factor; **CF**, coelomic fluid; **EFE**, earthworm fibrinolytic enzyme; **PAMP**, pathogen associated molecular pattern; **PRR**, pattern recognition receptor; **TLR**, Toll-like receptor; **tPA**, tissue plasminogen activator

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INTRODUCTION

All invertebrate animals, as well as earthworms, express various immune mechanisms against pathogens. These mechanisms are accomplished through cellular and humoral immunity, similar to those of the vertebrate immune system. The innate immunity in earthworms is maintained by cellular components, different coelomocytes (leukocytes), housed in coelomic cavity. The fluid from coelomic cavity (CF) also contains many other immunologicaly active (antimicrobial) molecules, such as lysenin, fetidin, eiseniapore, coelomic cytolytic factor (CCF-1), Lumbricin I, etc. (Cooper *et al*. 2002).

The use of the earthworms for medicinal purposes was first documented, back in 1340 A.D (Stevenson 1930; Reynolds *et al*. 1972). They have also been used for the treatment of various diseases by North American Indians and doctors in East Asia. Traditional Chinese medicine has also recognized the healing properties of the earthworms a long time ago (Cooper *et al*. 2004). The research of pharmaceutical effects of the earthworms has been initiated along with the development of biochemical technologies. The fibrinolytic enzymes were first isolated from earthworms in 1980's (Mihara *et al*. 1983; Lu *et al*. 1988; Hrženjak *et al*. 1991; Lin *et al*. 2000), and since then, the medical value of earthworms has been investigated in more detail. It was found that the earthworm extracts significantly diminish the coagulation of platelets and promote the dissolving of thrombi in the blood. Its therapeutic and preventive effects for thrombosis-related diseases have been clinically confirmed. After oral administration of the earthworm fibrinolytic enzyme (EFE) to the patients, reduction of fibrin and blood platelets coagulation has been noted. Moreover, there was no adverse effect on the functions of the nervous system as well as, respiratory system, cardiovascular vessels, liver and kidneys (Stein *et al*. 1982; Valenbois *et al*. 1982; Hirigoyenberry *et al*. 1990; Cho *et al*. 1998). The previous studies indicate that the coelomic fluid of earthworms also exhibits other biological functions, such as bacteriostatic (Cooper *et al*. 2004; Popović *et al*. 2005), proteolytic (Nakajima *et al*. 1993; Wang *et al*. 2003), cytolytic (hemolytic) (Popović *et al*. 2001; Procházková *et al*. 2006; Mataušić-Pišl *et al*. 2011), and mitogen activity (Hrženjak *et al*. 1993).

The agents with biomedical potential, prepared or isolated from the earthworms, have also been tested in wound

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healing. The characteristics of earthworm's homogenate/paste, which could contribute to better healing of wounds, have been reported. (Hrženjak *et al*. 1993; Hrženjak *et al*. 1998; Popović *et al*. 2001; Grdiša *et al*. 2001; Cooper *et al*. 2004; Grdiša *et al*. 2004; Popović *et al*. 2005; Balamurugan *et al*. 2007; Prakash *et al*. 2007). The activities like mitogen, antibacterial, haemostatic and antioxidative, mainly contribute to the healing and epithelization of wound. However, the paste obtained from earthworm *L. mauritii* Kinberg, exhibited variety of activities, such as antiinflammatory, antioxidative and hepatoprotective (Balamurugan *et al*. 2007; Prakash *et al*. 2007). According to the properties of animal extracts, they should be considered for the treatments of the wound as well as different human disease. This review summarizes some knowledge about the therapeutic properties of macromolecules from the earthworms.

IMMUNE SYSTEM IN EARTHWORMS

The earthworm (phylum Annelida, family Lumbricidae) is one of the first organisms in the evolution of any phylogenetic tree that possess immunological recognition and memory. Their immunes system has been characterized as an innate immunity, and some functions associated with the adaptive immunity have been documented, such as allogenic tissue rejection (Cooper *et al*. 1995, 1999). The universal key components of innate immune systems are the pattern recognition receptor (PRR) which recognizes pathogen associated molecular pattern (PAMP). The PAMPs are conserved structures of the proteins and nucleic acids found in the viruses, bacteria and fungi. In the mammalian innate immune system the PRRs are Toll-like receptors (TLRs) and they play the central role (Imier *et al*. 2004). In the case of the earthworms only few PRRs have been identified, while no TLRs (Engelmann *et al*. 2005; Cooper *et al*. 2006). The coelomocytes play a central role in the earthworm immune system. They are involved in immune functions characteristic of innate immunity such as phagocytosis and release of lytic factors (CCF-1). CCF-1 is a PRR and performs several immune functions, including cell lysis and binding to the pathogens via PAMPs (Beshin *et al*. 2002). The earthworm coelomocyte cells also provide immune functions and possess several CD markers (CD11, CD24, CD45RA, CD45RO, CD49b, CD54 and CD90) associated with innate immunity. Thus, they are the potential candidates for procession of TLR (Cossarizza *et al*. 1995, 1996; Cooper *et al*. 2002; Engelmann *et al*. 2002, 2005). The coelomocytes are involved in eliminating of foreign material by phagocytosis, encapsulation and NK-like activity (Stein *et al*. 1977, 1981; Cossarizza *et al*. 1996). They synthesized and secrete immuno-protective molecules into the CF, causing agglutination, opsonisation and lysis of foreign material. However, they are also involved in clotting reactions and phenoloxidase cascade (Cooper *et al*. 2002). A foreign material induces the synthesis of immune proteins in earthworms, which results in increased levels of agglutinins and lysins, proteases, antimicrobial molecules and other enzymes (Mohrig *et al*. 1996). The functions of agglutinins are aggregation and immobilization of foreign material, promotion of its phagocytosis and encapsulation, which leads to its elimination. The target recognition moieties for earthworm agglutinins and lysins are carbohydrates, so called lectins (Kauschke *et al*. 2000). Very important factors in the immune system of invertebrates and vertebrates are the proteases which contribute to the destruction of foreign materials in general. The proteases play an important role in invertebrates in a process of clot formation and activation of enzyme cascades (complement activation, prophenoloxidase-cascade) (Söderhäll and Cerenius 1998) as well as in degradation of foreign material. The patterns of CF protease can be considered as species specific in earthworms (Mohrig *et al*. 1989; Kauschke *et al*. 1997, 2000) and are affected by non-self-challenging. CF protease patterns might be seen as promising biomarker candidates in environmental monitoring studies. The results of disturbing homeostasis in earthworms are an increased immune response, which is reflected in increased activity of easily measurable humoral immune factors like agglutinins, lysins and proteases. Among them the protease pattern and the activity level may be consider as the best candidate in environmental monitoring (Kauschke *et al*. 2007).

EARTHWORM'S ENZYMES AS FIBRINOLYTIC AND ANTICOAGULATIVE AGENTS

In traditional Chinese medicine the earthworms have been used to improve blood circulation, to treat apoplectic stroke, and as antipyretic and diuretic agents. The earthworm fibrinolytic enzyme (EFE) is a complex protein enzyme that is widely distributed in the earthworm's digestive cavity. So far the variety of EFEs was isolated and characterized from different earthworm species, e.g. *L. rubellus* (Mihara *et al*. 1983; Nakajima *et al*. 1993), *L. bimastus* (Cheng *et al*. 1996; Xu *et al*. 2002) and *E. foetida* (Zhou *et al*. 1988; Hrženjak *et al*. 1998; Wang *et al*. 2003; Li *et al*. 2003). Most earthworm fibrinolytic enzymes showed distinctive high stability and strong tolerance to organic solvents and high temperature. Due to their fibrinolytic activity in dissolving fibrin in blood clots, they could be used in the treatment of cardiac and cerebro-vascular diseases. Besides the strong protein hydrolysis activity with direct effects on fibrin, EEF can also activate plasminogen. Clinical experiments showed that fibrinolytic enzymes after oral administration to the patients suffering from thrombosis could reduce coagulation of fibrin and blood platelets, without any side effects on other functions (Ryu *et al*. 1994; Lijnen *et al*. 1995; Gao *et al*. 1999: Zheng *et al*. 2000). Thus, the fibrinolytic enzymes from earthworms can be considered as safe and effective agents for the dissolving of the fibrin clots in the treatment of thrombosis, cardiac and cerebro-vascular clotting diseases.

These enzymes belong to serine protease family with fibrinolytic activity (Zhou *et al*. 1988; Nakajima *et al*. 1993; Sumi *et al*. 1993; Hrženjak *et al*. 1998). Chemical modification of these enzymes has been performed for parenteral administration (Nakajima *et al*. 1993; Wu *et al*. 2002). Comprehensive clinical trials have also been pursued. The most remarkable feature of these enzymes is the way of their absorption. The EFE-3, isolated from *L. rubellus*, could be transported into blood through intestinal epithelium where it exerts its biological function in circulation (Fan *et al*. 2001). Other fibrinolytic enzymes such as urokinase and tissue plasminogen activator can only be used by intraperitoneal injection rather than oral administration.

The potential use of fibrinolytic enzymes in the prevention and treatment of serious cardiac and cerebro-vascular diseases has been very attractive in medicine and pharmacology. The main cause of cardiovascular disease is intravascular thrombosis due to fibrin aggregation. Myocardial infarction is the most common form of such thrombosis. The primary protein component of blood clots is fibrin. The blood clots are formed from fibrinogen by thrombin (Voet and Voet 1990). The insoluble fibrin fibers are hydrolyzed into fibrin degradation products by plasmin, which is generated from plasminogen by plasminogen activators such as tissue plasminogen activator (t-PA), vascular plasminogen activator, blood plasminogen activator, urokinase, Hageman factor, and streptokinase plasminogen complex (Collen and Lijnene 2001). Normally, the formation and fibrinolysis of fibrin clot is well balanced in biological systems. However, the absence of fibrin hydrolyzation, due to some disorder, may cause thrombosis. Thus, the search for fibrinolytic reagents from different sources is of great interest and in progress.

The liver plays a central role in haemostasis by synthesizing most coagulation factors, coagulation inhibitors, fibrinolytic proteins and their inhibitors. The reticuloendothelial system of the liver is responsible for clearing all activated clotting factors such as the activation complexes of both coagulation and fibrinolysis and the degradation products of fibrin and fibrinogen (Brohy *et al*. 1996). The patients who suffer from liver disease may develop a wide spectrum of coagulopathy/hyperfibrionlysis.

Recently it was found that the fibrinolytic enzyme from *E. foetida* Protease-III-1 (EfP-III-1) acts in both, fibrinogenolysis and fibrinogenesis (Zhao *et al*. 2007). This enzyme hydrolysed fibrinogen and activated plasminogen and prothrombin. The activation of plasminogen and releasing active plasmin suggested on tPA-like function of EfP-III-1. Furthermore, EfP-III-1 showed a factor Xa-like function on prothrombin, producing alpha thrombin. Thus, EfP-III-1 may play an important role in the balance between procoagulation and anticoagulation. In recent years, the mixture of earthworm proteases, including EfP-III-1 has been made as an orally administrated fibrinolytic agent to prevent and treat clotting diseases (Califf *et al*. 1988; Sumi *et al*. 1993; Kim *et al*. 1998), with relatively low side effects (Sun and Fan 1998). The enzyme also exhibited the activity of fibronectinase (FNase) and cleaved fibronectine (FN) much faster than other proteins in serum. Since fibronectine has an important function in virus-binding activity, the FNase from *E. foetida* could be a good candidate for therapeutic treatment of hepatitis virus infection (Wang *et al*. 2008).

Clinical study of cerebral infarction has shown that earthworm proteases decrease some stroke scores in comparison with control group (Sun and Fan 1998). According to clinical observations (Tracy *et al*. 1985; Cannon 1995; Sun and Fan 1998), the activated partial thromboplastic time was prolonged, tPA activity and D-dimer levels increased, and the concentration of fibrinogen in blood decreased significantly.

The influence of G-90 treatment on haematological and haemostatic parameters was monitored *in vivo* on the rats (Matаušić-Pišl *et al*. 2011). The results have shown the most pronounced effect of G-90 to be exerted on bleeding and coagulation time, thrombin time and plasminogen level. The results have shown the influence of G-90 on blood coagulation to be very similar to that of heparin, a known anticoagulant. Thus, G-90 could be considered as a new thrombolytic agent of use in veterinary and human medicine.

The fibrinolytic enzymes could find a place in pharmaceutical industry as the agents for treatment of deregulated haemostasis, for prevention of blood clots formation and for the balance of fibrinolysis. The earthworms are very appropriate organism, found all over and the preparation of their extracts is usually very simple (G-90). However, the problem of these extract is low concentration of pure enzyme, responsible for fibrinolytic activity. Relatively low yield in the process of purification of these enzymes by biochemical approaches is the major limitation for their clinical use as therapeutic agents. Recently, certain progress has been made towards production of fibrinolytic enzymes via genetic engineering (Sugimoto *et al*. 2001; Hu *et al*. 2005; Yuan *et al*. 2006; Li *et al*. 2008). These approaches could give the advantage of using the earthworm enzymes as safe anticoagulants and fibrinolytic agents.

ANTITUMOR ACTIVITY OF THE EARTHWORM EXTRACTS

There has also been increased interest in the antitumor activity of EFE. The antitumor activity of EFE isolated from *E. foetida* was evaluated on human hepatoma cells *in vitro* and *in vivo* (Chen *et al*. 2007). Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer related mortality worldwide (Sherman and Takayama 2004). EFE showed significant antitumor activity in hepatoma cells, both *in vivo* and *in vitro*, perhaps through induction of apoptosis. These results pointed that EFE could be used in treatment of hepatoma. Similar antitumor effect of earthworm extracts has been noticed earlier (Chen *et al*. 2001; Hu *et al*. 2002; Xie *et al*. 2003; Yuan *et al*. 2004).

Antitumor effect of the earthworm proteases has also been reported. Macromolecular mixture (G-90), obtained from the tissue homogenate of *E. foetida* exhibited the antitumor activity *in vitro* and *in vivo* (Hrženjak *et al*. 1993). Such effect has been seen with coelomic fluid of *E. foetida*. Isolated coelomic cytolytic factor 1 (CCF-1) was capable of lysing different mammalian tumor cell lines (Bilej *et al*. 1995).

ANTIBACTERIAL PROPERTIES OF THE EARTHWORMS

In recent years the interest in antimicrobial peptide increased. They served as a first line defense against microbial invasion, supplementing the host's humoral and immune system. The earthworms, as well as other invertebrates, do not produce specific antibodies and they rely on innate mechanism for host protection against microbial attacks. Thus, the earthworms have developed an efficient defense mechanism against invading microorganism, which threaten their existence. Such defenses are present in coelomic fluid of earthworms (*L. rubellus* and *E. foetida* (Stein *et al*. 1982; Valenbois *et al*. 1982). This activity is attributed to the proteins including lysozime-like molecules and factor with haemolytic activity, as well as a pattern recognition protein named coelomic cytolytic factor (CCF) (Hirigoyenberry *et al*. 1990; Milochau *et al*. 1997; Cho *et al*. 1998). However, it has been shown that glycolipoprotein mixture (G-90) from *E. foetida* exhibited strong antibacterial activity against non-pathogenic and facultative-pathogenic bacteria (Popović *et al*. 2005).

Six antimicrobial peptides from earthworm tissue liquid homogenate and coelomic fluid have been isolated and purified (Wang *et al*. 2007). The peptides contained 5-50 amino acid residues with the same or similar sequence of Ala-Met-Val-Ser-Gly, named antibacterial vermipeptides family (AVPF). The AVPF exhibited wide antibacterial activity, including the Gram-positive and Gram-negative bacteria as well as the fungi.

EARTHWORM'S MACROMOLECULES IN WOUND HEALING

Mitogenic, antibacterial, haemostatic and antioxidative properties determined in earthworms, have a major influence on wound healing and epithelization. The use of natural products derived from plants or animal sources, offers the possibility of exercising a new approach both in comparative and alternative medicine settings. The implementation of some of these compounds into the treatment of human and animal diseases might as well be set as the goal that both scientists and experts engaged into comparative and alternative medicine should strive to achieve.

Fairly recently, the earthworm paste (*L. mauritii* Kinberg) has been launched, exhibiting antiinflammatory and antioxidative properties and influencing haematological parameters, all of the aforementioned being important for the wound healing process (Balamurugan *et al*. 2007; Prakash *et al*. 2007).

The earthworm preparations from *L. rubellus* and *E. foetida* promoted wound healing (Li *et al*. 2000; Matаušić-Pišl *et al*. 2010). Both preparations shortened the healing time by increasing epithelization, granulation and synthesis of collagen.

ANTIPYRETIC AND ANTIOXIDATIVE ACTIVITIES IN EARTHWORMS

Antipyretic activity has been detected in the earthworms *Lumbricus* spp. and *Perichaeta* spp. (Hori *et al*. 1974), as well as in paste obtained from earthworm *L. mauritii* Kinberg (Balamurugan *et al*. 2007). This activity was similar to that obtained with aspirin (Ismail *et al*. 1992). The antipyretic and antioxidative properties of paste from *L. mauritii* have also shown promising results in the treatment of peptic ulcer in rats (Prakash *et al*. 2007). The hepatoprotective potential of extract from *L. mauritii* has been noticed, after paracetamol-induced liver injury in Wistar rats (Bala-

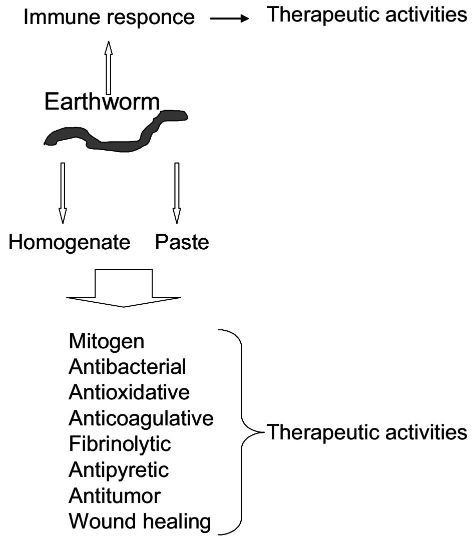

Fig. 1 The properties of earthworm macromolecules which contribute to their therapeutic activities.

murugan *et al*. 2008).

Natural antioxidants protect the human body from free radicals and retain the progress of many chronic diseases. Non-enzymatic antioxidants such as glutathione, vitamins C and E, Tocopherol and Ceruloplasmin protect the cells from oxidative damage (Aldrige 1981). The enzymatic antioxidants, such as superoxide dismutase, catalase and cyclooxigenase protect the cells from lipid peroxidation and they are very important scavengers of superoxide ion and hydrogen peroxide (Scott *et al*. 1991). The antioxidative activity has been detected in different preparations of earthworms (Grdiša *et al*. 2001; Balamurugan *et al*. 2007).

CONCLUSIONS

Earthworms have been used as a drug to improve blood circulation for centuries. The interest for naturally accessible therapeutic agents has been increasing in time. Thus, the earthworms with wide variety of biologically active components (**Fig. 1**) are very important for pharmaceutical industry. Their preparations are very suitable for the treatment of various diseases due to low cost, easy preservation, and without any toxic and side-effects.

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