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Unearthing a source of medicinal molecules

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Bioprospecting introduced enormous promise of additional sources of beneficial products that now confirm the ancient practices associated with Ayurveda (India), TCM (China) and Kampo (Japan).

With the surge of immunology in the early 1960s, there was a need for a more encompassing view revealing the pervasiveness of the immune system demonstrated as rejection of transplants in earthworms. Analogous with the importance of natural products derived from plants, it was clear that similar useful natural substances might be found in worms. Fortunately, marine animals emerged and so did the earthworm, the humoral immune systems of which provided components useful in current approaches to complementary and alternative therapies. This introduced the enormous promise of additional sources of beneficial products now confirming ancient practices: Ayurveda (India), traditional Chinese medicine (China) and Kampo (Japan). This review provides basic knowledge on drug discovery from the earthworm.

My aim as a biologist was to challenge the anthropocentric view that immunity was purely mammalian there for human [1–6]. What emerged and is widely accepted is the universality of innate immunity of the cellular and humoral type—essential throughout the living world and necessary for the full initiation and completion of the adaptive immune response that vertebrates, including humans, inherited. The importance of natural products derived from plants and animals when analyzed in appropriate assays could be shown to be relevant in our quest for defining useful treatments applicable to human maladies. Sources such as marine species emerged – and so did the earthworm – as animals whose humoral immune systems provided the fodder for components in earlier and even current approaches to complementary and alternative therapies. Thus, bioprospecting came on the scene.

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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, from convulsions and fevers to rheumatoid arthritis and blood stasis syndromes [7–15]. Earthworms are also used in treating blepharoptosis, or drooping of the upper eye lid, along with other phlegm herbs (e.g. Dan Nan Xing, Jiang Can, Ban Xia, Tian Ma and Bai Fu Zi) (Figure 1). Di Long comes in two variants, Guang Di Long (native to Guangdong, Guangxi and Fujian and collected from spring to autumn) and 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. According to TCM, Di Long is associated with the bladder, liver, lung and spleen meridians and has salty and cold properties. Di Long 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 habits of burrowing through the earth, constantly searching out new spaces in which to slither. In nutritive components, Di Long can 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.

TCM: chemical ingredients for Di Long, the common name for the earthworm *Lumbricus*

The earthworm, an ancient Ayurvedic source

From another ancient culture, in India, within the Ayurvedic tradition, there are similar practices to those in China (Dilong is

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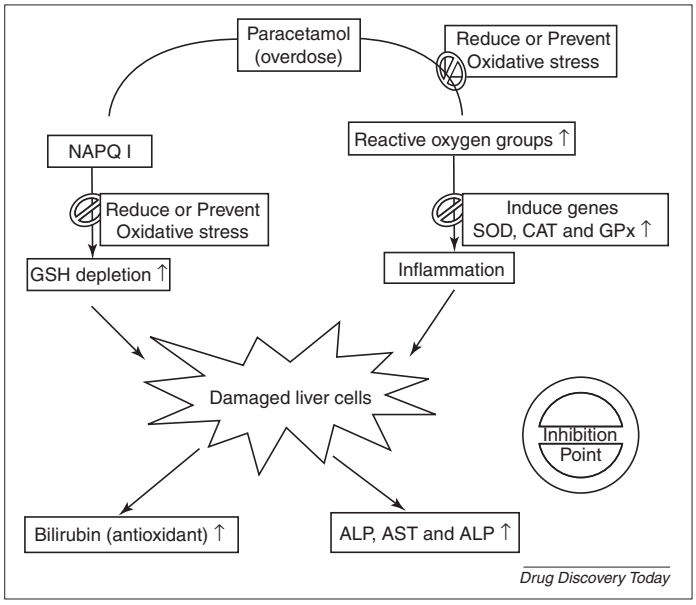

FIGURE 1

How does earthworm extract ameliorate liver damage?

a transliteration of the Chinese term for the animal itself). According to Cooper *et al.* [16], indigenous people throughout the world, more particularly in Asia (including India, Myanmar, China, Korea and Vietnam) have traditionally practiced extracting and using biologically active compounds from earthworms [17]. Table 1 shows the activities of some of these compounds with fibrinolytic activity. Table 2 shows a more varied functional diversity, including – for example – mitogenicity, hemolytic hemagglutination, perforin-like activity, hemolysis, cytotoxicity, vasodepressor and antibacterial activity. Earthworms have a dense nutritional content because of their soil-based origin. Previous studies, using earthworms in the context of vermibiotechnology, have shown their antipyretic, antispasmodic, detoxic, diuretic, antihypertensive, antiallergic, antiasthmatic, spermatocidal, antioxidative, antimicrobial, anticancer, antiulceral and anti-inflammatory activities [4,5,18–21]. This, of course, reminds us of the earthworm's innate immune properties. In the first of several experiments, in rats whose livers have been damaged by paracetamol, earthworm extracts (EEs) were involved in a hepatoprotective role, whether by preventing damage owing to oxidative stress, by enhancing antioxidant activity, by reducing lipid peroxidation or by all these mechanisms.

Antiulcer and antioxidative therapeutic properties

Lampito mauritii (Kinberg), an indigenous earthworm species widely used in Siddha and Ayurveda, possesses anti-inflammatory, antiulceral and antioxidative properties. To test and confirm this assertion, Prakash and Ranganathan [20] have analyzed the antiulceral and antioxidant properties of 'earthworm paste' (EPA) derived from *Lampito mauritii*. EPA effects were compared with a standard antiulceral drug, ranitidine, on the Wistar strain albino. Controls, administration of 200 mg/kg aspirin, increased the volume of gastric juice secretion, total acidity, free acidity and ulcer index and reduced the pH. Experimental animals also had decreased antioxidant levels (i.e. reduced glutathione, glutathione peroxidase, catalase and superoxide dismutase) but increased levels of thiobarbituric-acid-reactive substances. Pre-treatment with the standard drug, ranitidine (50 mg/kg) and different doses of EPA (20, 40, 80, 160 and 320 mg/kg) in rats with induced ulcers enhanced the pH, decreased the volume of gastric juice, free acidity and total acidity and reduced the ulcer index. Moreover, activities of reduced glutathione, glutathione peroxidase, catalase and superoxide dismutase were increased, whereas the thiobarbituric-acid-reactive substance decreased. Results were more considerable in rats administered with 160 mg/kg EPA than by the application of ranitidine and other doses of EPA. These results suggest that EPA possesses antiulcer and antioxidative therapeutic properties.

Anti-inflammatory activity and antioxidant property associated with high polyphenolic content. After these encouraging results, a newer challenge began an analysis aiming to understand more clearly those therapeutic properties, such as anti-inflammatory, antioxidative, hematological and serum biochemical markers, associated with EPA [20]. Initially, investigators compared EPA activity with the standard anti-inflammatory drug, aspirin, on rats. Administration of EPA (80 mg/kg) to albino rats that had been previously induced to present an inflammatory response showed reduced inflammation and restored levels of antioxidants but reduced

TABLE 1

Therapeutic properties of earthworms (fibrinolytic)

Earthworm	Active fraction	Activity	Refs
Eisenia fetida	G-90	Fibrinolytic activity against malignant tumor patients' blood	[44]
Pheretima	Fibrinolytic enzyme	Fibrinolytic	[45]
Lumbricus rubellus	Freeze-dried powder	Antithrombotic and fibrinolytic	[31]
E. fetida	Glycosylated fibrinolytic enzyme	Fibrinolytic	[46]
E. fetida	Fibrinolytic enzymes extracted from earthworms reared in different substrates	Fibrinolytic	[47]
L. rubellus	Fibrinolytic enzymes	Fibrinolytic	[48]
L. rubellus	Fibrinolytic protease (F-III-2)	Fibrinolytic	[49]
L. rubellus	Serine protease	Fibrinolytic	[50]
E. fetida	Fibrinolytic enzyme (eFE-D)	Fibrinolytic	[51]
E. fetida	SDS-activated fibrinolytic enzyme	Fibrinolytic	[52]
E. fetida	Earthworm protease-II and III	Fibrinolytic	[53]

TABLE 2

Therapeutic properties of earthworms (non-fibrinolytic)

Earthworm	Active fraction	Activity	Refs
Eisenia fetida	Coelomic Cytolytic Factor-1 (CCF-1)	Cytolytic	[54]
E. fetida	G-90	Mitogenicity	[55]
E. fetida and *Lumbricus terrestris*	Coelomic fluid	Hemolytic and hemagglutinating	[56]
E. fetida	Small coelomocytes	Perforin-like activity	[57]
E. fetida	Lysenin	Hemolysis, cytotoxicity and vasodepressor	[58]
E. fetida	Eiseniapore	Cytolytic	[59]
E. fetida	Fetidins	Antibacterial Hemolytic and hemagglutinating	[60]
E. fetida, L. terrestris and *Aporrectodea caliginosa*	Hemolytic and hemagglutinating proteins in the coelomic fluid	Hemolytic and hemagglutinating	[61]
Eisenia fetida andrei	Eisenia fetida andrei factor	Hemolytic activity	[62]
E. fetida andrei	Hemolysins	Hemolytic	[63]

glutathione, glutathione peroxidase, superoxide dismutase, catalase and thiobarbituric-acid-reactive substances. Moreover, at the cellular level, treated rats showed restoration to normal values of erythrocytes and leukocytes. More specifically, differential levels of neutrophils, lymphocytes and eosinophils were restored, as were hemoglobin and serum biochemical components. These included protein, albumin, glucose, cholesterol, and acid and alkaline phosphatase and electrolytes (e.g. sodium, potassium and chloride). According to one interpretation, the anti-inflammatory activity together with antioxidant property of EPA seems to be due to the high polyphenolic content of earthworm tissues; polyphenolic compounds have been extensively studied and have shown great potential as antioxidants. Because they are present in the EE, the antioxidant properties of EEs might be a result of the component polyphenols.

Restoration of liver histoarchitecture. Developing on previous studies, Balamurugan [21] demonstrated that EE was capable of concomitantly restoring histoarchitecture in paracetamol-induced liver damage in rats. Whole-tissue extract provided dose-dependent (over a range of 100–300 mg/kg body weight) liver protection to rats given paracetamol at 2 g/kg to induce liver damage. The treatment produced a statistically significant reduction ($P < 0.05$) for the hepatic marker enzymes aspartate transaminase, alanine transaminase and alkaline phosphatase that was similar to silymarin administered at 150 mg/kg. Histopathological observations of liver tissues confirmed such biochemical evidence.

Anti-inflammatory and antipyretic activities of EE

EE stimulated properties similar to glycolipoprotein complex (G-90). Administration of indomethacin (10 mg/kg), paracetamol (150 mg/kg) and/or various doses of EE (50, 100 and 200 mg/kg) were all capable of reversing histamine-induced and turpentine-induced inflammation (in both acute and chronic phase) and Brewer's yeast-induced pyrexia in rats, effectively back to normal [20].

Modes of action

Hepatoprotective and antioxidant properties

Balamurugan *et al*. [22] have suggested that the hepatoprotective properties observed in paracetamol-induced liver injury in rats might have a similar mode of action to silymarin, a standard hepatoprotective drug. Consequences of liver injury include eduction in liver antioxidants (such as glutathione, superoxide dismutase, glutathione peroxidase and catalase), as well as reduced serum total protein, and increased serum components such as alkaline phosphatase, aspartate aminotranferase (AST), alanine aminotranferase (ALT), bilirubin and liver thiobarbituric-acid-reactive substances. Administration of EE (100, 200 and 300 mg/kg) increased activities of liver glutathione, superoxide dismutase, glutathione peroxidase and catalase and elevated serum total protein levels but resulted in decreased levels of serum alkaline phosphatase, AST, ALT, bilirubin and liver thiobarbituric-acid-reactive substances. The changes in these liver markers were similar to those produced by the hepatoprotective drug silymarin at a dose of 150 mg/kg. The mode of action of EE suggests that it might prevent the formation of reactive oxygen species, or that EE might assist in reactive oxygen species scavenging. As a result, EE prevents damage to hepatic cells, modulates the genes responsible for synthesis of antioxidant enzymes (glutathione peroxidase, catalase and superoxide dismutase in liver tissue), and decreases serum enzymatic activities such as alkaline phosphatase, AST and ALT.

Inter-species properties

By way of confirmation, extension and validation of these observations, Prakesh used another earthworm species (*Perionyx excavatus*) to examine certain pharmaceutical effects [23]. Analyses focused on the hepatoprotective and antioxidant properties of earthworm powder (EPO), not EE or EPA, in a model of alcohol-induced hepatotoxicity. Rats with alcohol-induced hepatotoxicity showed elevation in the lipid-peroxidative marker, thiobarbituric-acid-reactive substance. There was a decrease in the activities of the antioxidant enzymes superoxide dismutase, catalase and glutathione peroxidase and in the non-enzymatic antioxidants vitamin C, vitamin E and reduced glutathione. Oral administration of dried EPO (500 mg/kg) for 42 days reversed these parameters towards normal levels. Results similar to those of others suggest that this indigenous earthworm could afford a considerable hepatoprotective and antioxidant effect in alcohol-induced hepatotoxicity in rats. What is even more crucial is that EPO seemed to be as effective as EE in altering the pathology induced by alcohol, thus potentially confirming a universality of effects caused by earthworm products.

BOX 1

Functional properties of lumbrokinase[a]

Leading researchers and doctors report on the power of lumbrokinase to:
Dissolve clots and protect against ischemic heart disease and stroke
Lower fribrinogen levels in cancer patients, which is strongly associated in scientific studies with better outcomes, less metastasis, and slower growth of tumors
Dissolve bacterial biofilms present in chronic infections in conditions like autism and Lyme disease, allowing antimicrobials to work effectively
Offer antiplatelet, anti-thrombotic and anti-apoptotic activity, remarkably regulating hypercoagulation

[a]Adapted from a table from Ref. [7].

Lumbrokinase from *Lumbricus rubellus*

Basic characteristics related to clinical trials

The earthworm has been used as a drug for various diseases in China and the Far East for thousands of years, although without rigorous scientific evaluation [6]. According to records in the most famous Chinese ancient medical publication, *Ben Cao Gang Mu* (*Compendium of Materia Medica*), the traditional medico material 'Di Long' (earthworm) was regarded as being effective in treating limb numbness and hemiplegia. Moreover, Di Long was also effective as an antipyretic, capable of sedation and able to improve blood circulation and ameliorate clot formation. According to Cooper [7], lumbrokinase (LK) is a member of a group of proteolytic enzymes, which include plasminogen activator and plasmin that has been isolated from a particular earthworm (Box 1). The plasminogen activator (e-PA) in LK is similar to the plasminogen activator (t-PA) from other species. This enzyme can only demonstrate thrombolytic activity in the presence of fibrin; therefore, LK has the advantage of not causing hemorrhage owing to hyperfibrinolysis during treatment, in contrast with either streptokinase or urokinase. According to acute and subacute toxicological experiments, there were no reported negative effects of LK on the nervous, cardiovascular, respiratory or blood systems of rats, rabbits or dogs. In addition, long-term damage to hepatic or renal systems could not be demonstrated in animal toxicological experiments. Embryonic development, similarly, was not affected by such treatment; there have been no reports of either teratogenesis or mutagenesis in embryonic rats.

Treatment of embolism

In experimental acute pulmonary artery embolism in rabbits, the embolus was labeled with ^{125}I and the radioactivity in blood tested at 0.5, 1.0, 2.0, 3.0 and 5.0 h after duodenal administration of LK. There was a marked dose-dependent increase in radioactivity in the blood at 3.0 and 5.0 h after administration. Inferior *vena cava* thrombosis tests in rats showed a reduction in thrombosis after rectal administration of LK. LK decreases fibrinogen, thus lowering blood viscosity and reducing platelet aggregation. LK has also been reported to be effective in treating and preventing ischemic cerebrovascular diseases and other embolic and thrombotic diseases: coronary, myocardial infarction, deafness, arterial sudden thrombosis of the central retinal vein, embolism in peripheral veins and pulmonary infarction [24–32].

Boluoke: fibrin-dissolving enzyme from earthworms that might be applicable to disseminated intravenous coagulation

Boluoke (LK) is the only fully researched oral enzyme that supports healthier blood. It shows great promise in supporting a healthy balance of coagulation and fibrinolysis in the body [7,33,34] (http://www.naturodoc.com/library/heart/boluoke.htm). The coagulation system is a complicated and highly regulated system. Hypercoagulation means the body is producing fibrin strands faster than it can break them down. When this happens, the fibrin can become deposited on capillary walls, impeding the delivery of oxygen and nutrients from the blood into tissues and waste chemicals from them out to the bloodstream. If this situation is sustained for a prolonged period, the body's tissues will gradually become hypoxic (lacking in oxygen) and malnourished. The end result is often an acidic tissue environment, pain, lack of energy and the decline or loss of organ functions. Hypercoagulation also predisposes a person to clot formation (as in strokes or heart attacks) in blood vessels if atherosclerosis is already present. Research has clearly shown that hypercoagulation is often present in patients with chronic illnesses. If hypercoagulation is not diagnosed, patients often do not improve, or they relapse easily. In meningitis, there is often associated purpura fulminans. LK could be a candidate for amelioration of this condition.

Applications of earthworm lysins to membranes

Relevance to human diseases

Bioprospecting upon careful analyses reveals useful products synthesized and secreted even by sponges—the first multicellular members of the second animal phylum, the Porifera [4,5]. If we turn now to more complex multicellular species for which there is substantial centuries-old information, witness the literature pertaining to the earthworm's healing properties [6]. Earthworm lytic molecules are antimicrobial and fibrinolytic and might prove useful as antibacterial agents and prophylactic molecules [24–32]. Two molecules, lysenin and eiseniapore, can target intracellular lipid-trafficking mechanisms. Trafficking dysfunction can directly produce pathology – for example, in Tangier disease and Niemann-Pick disease type C – or might be able to contribute to pathology in diseases such as Alzheimer's and atherosclerosis. Lysenin reacts specifically with fibroblast membranes from patients with Niemann-Pick disease, a rather curious finding, but one that might have some clinical relevance [35,36] (Table 3). Niemann-Pick diseases are genetic diseases grouped under sphingolipidoses, or lipid storage disorders. For them, excessive lipids accumulate in the spleen, liver, lungs, bone marrow and brain. There is a classic infantile type A variant that causes complete deficiency of sphingomyelinase. Sphingomyelin is a component of cell membrane, including the organellar membranes; thus, the enzyme deficiency blocks degradation of lipid. This results in the accumulation of sphingomyelin within lysosomes in the macrophage-monocyte phagocyte lineage (the body's phagocytic cells). When cells are affected, they often become enlarged, approximately 90 microns in diameter. Microscopic analysis reveals lipid-laden macrophages in the bone marrow, whereas pathologic examination shows 'sea-blue histiocytes'. Numerous small vacuoles of uniform size are created.

TABLE 3

Peptides derived from earthworm that mediate cytolysis[a,b]

Nomenclature: molecular weight and investigators	Accession number	Homology	Site of expression	Proposed function
Lysenin EfL1 (L1) EfL2 (L2) EfL3 (L3) 42-kDa Sekizawa *et al.*, 1997 *Gene* Fetidin [64]	D85846 D85847 D85848	U02710 (Fetidin 1)	Coelomic fluid; chlorogocytes; coelomocytes	Contracts rat smooth muscle
Fetidin 1 40-kDa Four isoforms Fetidin 2 45-kDa Monomorphic Lassegues *et al.*, 1997 [65] *Eu J Biochem* Coelomic cytolytic factor 1	U02710	D85848 of (Lysenin)	Coelomic fluid; chlorogocytes; inducible; increases after injecting pathogenic bacteria	Hemolysis; bacteriolysis; agglutination; clotting; opsonization; heme-binding enzymes; horseradish peroxidase; protein U1 32; *herpes simplex virus*
CCF-1 42-kDa Beschin *et al.*, 1998 [66] *J Biol Chem* Lumbricin	AF030028	AF395805 of (CCF precursor)	Coelomic fluid β-1, 3-glucan LPS	Cytolysis; opsonization; hemolysis
Lumbricin I 7.2-kDa Cho *et al.*, 1998 [67] *Biochem Biophys Acta*	AF060552		Whole worm; not inducible; constitutive	Antimicrobial; not hemolytic; not induced by infection

[a] All derived from *Eisenia foetida*, except antimicrobial Lumbricin I from *Lumbricus rubellus*.
[b] Modified from Bioessay 24.4 Cooper *et al.* 2002 [52].

This gives a foamy appearance to the cytoplasm. The relevance and curiosity derives from the finding that fibroblast membranes can be recognized specifically by lysenin derived from earthworms (Table 3).

Specific binding of lysenin and sphingomyelin

Specific binding of lysenin to sphingomyelin on cellular membranes might prove to be a useful tool to probe the molecular motion and function of sphingomyelin in biological membranes, especially in an effort to explain the mechanism of lysis caused by earthworm products. These results emphasize simultaneously the need for expanded analyses of various lytic pathways that might be mediated by and within the context of the earthworm's immunodefense system. The products of sponges and those of other animals have potential importance that is at least considerable as that of the products being exploited from plants. One future goal will be to successfully introduce some of these compounds as treatments for human diseases. Such an approach would raise public awareness concerning the richness and diversity of natural products that could be carefully harvested for human benefit without damaging ecosystems. Serendipity has won again: how scientific approaches reveal solid evidence that certain molecules might now be ready for testing.

In vitro approach confirms in vivo outcome

According to Chu *et al*. [37], *Pheretima* (family *Megascolecidae*) have been documented as a potent agent for the treatment of cough and breathing difficulty in traditional Chinese medicine for nearly 2000 years. The water extract of *Pheretima* was separated into three fractions: the ethanolic precipitate fraction, the alkaline fraction and the acidic fraction. Of the three fractions, the acidic fraction showed the most potent spasmolytic effects on histamine-induced contractions in isolated guinea pig tracheal rings. In addition, it inhibits increase of short circuit current induced by carbachol in isolated rat tracheal epitheliums with the IC_{50} values of 0.15 and 0.08 mg/ml, respectively. A short circuit is an abnormal low-resistance connection between two nodes of an electrical circuit that are meant to be at different voltages, as applied. The half-maximal inhibitory concentration (IC_{50}) is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. This quantitative measure indicates how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biological process or component of a process (i.e. an enzyme, cell, cell receptor or microorganism) by half. Further *in vivo* studies concluded that the acidic fraction could protect an experimental asthma model induced by the combination of histamine and acetylcholine chloride in guinea pigs by prolonging the latent periods of asthma ($P < 0.05$) and significantly decreasing the cough frequency caused by ammonia water in mice ($P < 0.001$). This approach offers new and different ways to analyze the potential of material derived from earthworms because they might affect aspects of immunologic function such as immediate hypersensitivity [37].

Concluding remarks: perspectives on drug discovery from the earth

This article has highlighted a substantial amount of information outlining the uses and potential modes of action of several

BOX 2

Hypercoagulation is often present in the following conditions[a]

Cancer
Diabetes
Fibromyalgia
Crohn's Disease
Lyme Disease
Multiple sclerosis
Meniere's Disease
Chronic Fatigue Syndrome
Chronic infections
Lupus
Gulf War Syndrome
Excessive heavy metal burden
Elevated serum fibrinogen
Elevated serum CRP
Elevated serum Lp[a]
Elevated homocysteine
Angina
Heart attack history
Transient ischemic attacks
Ischemic stroke history
On birth control pills
On hormone replacement therapy
Thrombocythemia
Deep venous thrombosis
Being on long air flights
Hip fracture
Ulcerative colitis
Polycythemia
Vascular dementia
Autism
ADD/ADHD
Habitual miscarriages
Infertility

[a]http://www.naturodoc.com/library/heart/boluoke.htm.

potentially interesting components of EEs and EPO. Primarily, model systems have been directed demonstrating the ability of such components to ameliorate aspects of the inflammatory process. According to Wallace [38] and Haefner [39], despite recent developments in combinatorial chemistry that can rapidly generate thousands of new chemicals, the pharmaceutical industry still relies heavily on a staggering array of as yet undiscovered possibilities from the natural environment. The terrestrial environment has been successfully mined for compounds and, currently, promising ones such as those from the earthworm – a vital animal from the earth – are being exploited, notably in the Far East.

These chemicals are structurally complex, thus challenging organic chemists with approaches that might mimic synthetic versatility. Later, these products could well yield entirely new classes of drugs that would be valuable in treating human disease. Experimental trials have been directed to aspects of inflammation and lytic responses associated with hypercoagulation of blood (Box 2). Applications are pertinent, especially where coagulation and the need for dissolution are imperative. We need breakthroughs, overtures, discoveries and innovations in the face of rising medical costs and the fact that a considerable fraction of the general public (at least in the USA) is underinsured and often disgruntled with modern-day Western medical practice. Clearly, the most prevalent of products from the earthworm consist of their role in providing lytic activity against a variety of pathological conditions [40–43]. The earthworm has been crucial to the essence of this review concerned with aspects of inflammation [68]. This review now extends and clarifies information presented earlier [69].

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