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Anti–elastase, anti–tyrosinase and matrix metalloproteinase–1 inhibitory activity of earthworm extracts as potential new anti–aging agent

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PEER REVIEW

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Comments

This is a valuable research work in which authors have demonstrated the anti–ageing activity of EE as ant–ageing agent. The activity was assessed based on the inhibitory effect of EE against elastase and MMP–1 activities.
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ABSTRACT

Objective: To examine whether earthworms of *Eisenia fetida, Lumbricus rubellus* and *Eudrilus eugeniae* extracts have elastase, tyrosinase and matrix metalloproteinase–1 (MMP–1) inhibitory activity.

Methods: The earthworms extract was screened for elastase, tyrosinase and MMP–1 inhibitory activity and compared with the positive controls. It was also evaluated for whitening and anti–wrinkle capacity.

Results: The extract showed significantly ($P<0.05$) good elastase and tyrosinase inhibition and excellent MMP–1 inhibition compared to N–Isobutyl–N–(4–methoxyphenylsulfonyl)–glycylhydroxamic acid.

Conclusions: Earthworms extract showed effective inhibition of tyrosinase, elastase and MMP–1 activities. Therefore, this experiment further rationalizes the traditional use of this worm extracts which may be useful as an anti–wrinkle agent.

KEYWORDS

Earthworms, Ageing, Elastase, Matrix metalloproteinase–1, Anti–wrinkle agent

1. Introduction

Aging of the skin can be divided into intrinsic and extrinsic aging. Intrinsic skin aging can be classified as natural aging and is stimulated by changes in skin elasticity while extrinsic aging is caused by the exposure of the skin to solar radiation causing photoaging[1]. Collagen is the most abundant protein in the extracellular matrix (ECM) of connective tissue in the human dermis[2]. It functions as an adherence to connective tissues[3], creating suitable cellular environments that are needed during development and morphogenesis[4]. Deterioration of this protein is crucial as it allows changes in shape, cell migration or tissue desorption that are critically required in tissue remodeling during embryonic development, growth or disease processes[4]. Elastase is a member of the chymotrypsin family of proteases which is primarily responsible for the breakdown of elastin. It is an important protein found within the ECM. It can cleave elastin as well as collagen, fibronectin and other ECM proteins. The most important functions of elastase and matrix metalloproteinases (MMPs) after wounding process are to dispose foreign proteins within the ECM during phagocytosis by neutrophils and enable tissue repair under normal conditions[5]. However, due to chronic ultraviolet exposure, collagen and elastase in the dermis will denature, leading to wrinkles and photoaging of the skin[6]. This process will induce the production

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of MMPs by activating intracellular signal transcription pathways, including p38 mitogen-activated protein kinase and c-Jun-N-terminal kinase[7]. MMPs are enzymes that structurally correspond to the matrix degrading process or specifically zinc dependent proteases which are associated with a variety of destructive processes, including inflammation, tumor invasion and aging of the skin[8]. There are five classes of MMP family and its grouped according to their substrate specificity and/or structure[9]. Among these members, MMP-1 is primarily responsible for the degradation of collagen in the photoaging process of human skin which is secreted from human skin fibroblasts[10]. Tissue inhibitors of metalloproteinases (TIMPs) counteract with MMPs by inhibiting its activity and then limiting the breakdown of ECM. An agitated balance or condition of MMPs and TIMPs can be found in different pathologic conditions such as cancer, rheumatoid arthiritis and periodontitis, which is very crucial in finding the balance between MMPs and TIMPs to maintain the integrity of healthy tissues[11,12]. Therefore, the inhibitors of elastase and MMP-1 enzymes can be potential to be cosmetic ingredients in combating skin aging due to their usefulness in preventing the loss of skin elasticity and sagging. Earthworms play a fundamental role in the restoration of degraded lands and metal-contaminated soils[13,14]. With a very low toxicity which were attested by their long popular use as a natural remedy in traditional Chinese medicine since ancient times in certain parts of Asia, the fresh and powdered earthworms have been used for treating diseases like flu, cancer, heart attack, asthma, bronchitis, wound and serves as anti-inflammatory agent. Also, the earthworms are regarded as a potential food source with nutrient value and protein content. Analysis of two species of earthworms namely *Andiorrhinus kuru* sp. and *Andiorrhinus motto*, showed that they are composed of protein, amino acids, fatty acids, 20 minerals and other trace elements[15]. So far, no study has shown side effects associated with the dried powder of earthworm extract where it has been administered 200 mg/kg orally to rats which demonstrated good anti-inflammatory activity against this tamine-induced inflammation compared to indometacin drug[16]. Furthermore, earthworm proteases have been prepared into enteric-coated capsules for clinical use in China for more than 10 years[17]. In the past few decades, research on pharmaceutical and pharmacology effects of earthworms has been initiated[18]. However, to date, the direct modulatory effect of earthworm extract (EE) on elastase, MMP-1 and tyrosinase activity has not been demonstrated. MMP-1 and collagen degradation is prominent in inflammatory skin diseases and photoaging. If EE is proven to be an elastase, tyrosinase and MMP-1 inhibitor, the extracts can be a potential ingredients for anti-photoaging in cosmetics. Therefore, the objective of this study is to examine whether extracts from the earthworms of *Eisenia fetida* (*E. fetida*), *Lumbricus rubellus* (*L. rubellus*) and *Eudrilus eugeniae* (*E. eugeniae*) have elastase, MMP-1, and tyrosinase inhibitory activity.

2. Materials and methods

2.1. Preparation of EE from *E. fetida*, *L. rubellus* and *E. eugeniae*

The earthworms were washed thoroughly with running water a few times to remove dirt, soil and humus[19]. They were then sprinkled with 2.5 g of citric acid to remove harmful materials such as cyanide, arsenic and ammonia and left for about 15 min. The earthworms were washed again with running water to remove the citric acid residues, and then submerged in deionized water for 1 h. They were homogenized by using a homogenizer (IKA T18 basic Ultra-Turrax) for 30 min at speed of 13.3 r/min. The homogenates were kept at −80 °C for 24 h before freeze drying to produce earthworm powder. Then, four volumes of cold acetone were added to one volume of earthworm powder. The mixture was mixed and kept for 10 min at −70 °C and 90 min at −20 °C, followed by centrifugation at 10 801 r/min for 15 min at 4 °C. The supernatant was carefully discharged to retain the pellet. The pellet was air-dried for 24 h at room temperature to eliminate any acetone residue which resulted in EEs.

2.2. Elastase inhibitory assay

Elastase inhibition activity was determined according to the method of Ju, *et al.* with some modifications[20]. A 100 μL of 0.2 mol/L tris-HCl buffer, 25 μL of 10 mmol/L N-(methoxysuccinyl)-ala-ala-pro-val-4-nitroanilide (MAAPVN) and 50 μL of sample were mixed and incubated for 15 min. Then, 25 μL of 0.3 units/mL elastase (optimum reactivity of the enzyme) was added and incubated for another 15 min. The inhibition rate was measured by microplate reader at 410 nm and calculated as follows:

Inhibition rate (%) $1-[(C-D)/(A-B)]\times 100$

Where, A=absorbance of blank after incubation; B=absorbance of test sample before incubation; C=absorbance of test sample after incubation; D=absorbance without test sample before incubation.

2.3. MMP-1 inhibitory assay

The assay was determined according to Maiti *et al.*[21], by diluting 1 μL inhibitor N-Isobutyl-N-(4-methoxyphenylsulfonyl)-glycylhydroxamic acid (NNGH) in 200 μL assay buffer consisting of 50 mmol/L 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid, 10 mmol/L $CaCl_2$, 0.05% Brij-35, and 1 mmol/L 5, 5′-dithiobis(2-nitrobenzoic acid). The substrate was then diluted in assay buffer as needed, 10 μL per well. The MMP-1 (interstitial collagenase, fibroblast collagenase, human, recombinant) enzymes were diluted in assay buffer, 20 μL per well. All mixtures were heated to room temperature shortly before the assay was performed. The assay buffer was pipetted into each well of the microplate as follows: blank (no MMP-1)=90 μL assay buffer, control (no inhibitor)=70 μL assay buffer, and inhibitor NNGH=50 μL assay buffer. The microplate was allowed to equilibrate to assay temperature at 37 °C. A volume of 20 μL MMP-1 were added to each well except for the blanks. The NNGH inhibitors (20 μL) were added to the inhibitor NNGH wells. The test inhibitor was added to the wells and the plate was incubated for 30 min at 37 °C so as to allow the inhibitor/enzyme to interact. Then 10 μL of substrate was added to each well and the plate was read continuously for 10 min at 1 min interval at 412 nm. Data acquisitions were done according to the manufacturer's instruction.

2.4. Tyrosinase inhibition assay

The method from Tomita *et al.*[22] was slightly modified. A pre-incubation mixture consisting of 1.8 mL of 0.1 mol/L phosphate buffer, pH 6.5, 0.6 mL of H_2O_2, 0.1 mL of the EE dissolved in deionized water solution and 0.1 mL of the aqueous solution of the mushroom tyrosinase (300 U/mL) was pre-incubated at 25 °C for 5 min. Then, 0.4 mL of 6.3 mmol/L levodopa was then added and the reaction is monitored at 475 nm for 100 seconds. L-cystine (8 mg/mL) was used as the positive control. The negative control contained deionized water instead of the sample.

3. Results

3.1. Elastase inhibition and MMP-1 inhibition capacity

The activities of elastase and MMP-1 inhibitions exhibited by EE are shown in Figure 1. Both assays were carried out at 10 mg/mL and this concentration gave optimum results compared to 5 and 20 mg/mL concentrations. The three earthworms' species exhibited good anti-elastase activity, where the inhibition rates of *E. fetida*, *L. rubellus* and *E. eugeniae* were 46.98%, 53% and 51.82%, respectively; however their values were lower than epigallocatechin gallate (84.79%). Epigallocatechin gallate was used as positive control due to its positive inhibition on collagenase enzyme, expression of mRNA stromelysin induced by IL-1β activities and protection from skin damage caused by UV rays[23]. Different profiles were observed for the anti-MMP-1 activities of earthworm where *E. eugeniae* extract showed significantly the highest inhibitory activity (81.42%) at 10 mg/mL concentration, comparable to NNGH (86.18%). The inhibitory rate for *L. rubellus* and *E. fetida* were 72.90% and 75.47%, respectively, which can be considered as good inhibitors.

3.2. Tyrosinase inhibition capacity

The effect on tyrosinase inhibition of EE using mushroom tyrosinase is depicted in Figure 2. The assays were conducted at concentrations of 0.25 mg/mL only as it gave optimum results compared to concentrations of 1 and 5 mg/mL during the enzyme activity optimization. Tyrosinase inhibitor, L-cysteine was used as the positive control. *E. fetida* showed 80.12% inhibition of tyrosinase which was superior to the EE. Meanwhile, the inhibitions of *L. rubellus* and *E. euginae* were almost similar at 71.28% and 72.02%, respectively. However, all extracts exhibited lower anti-tyrosinase activity compared to L-cysteine (93.51%).

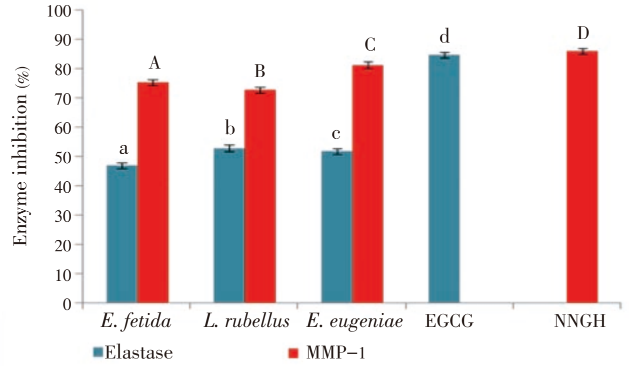

Figure 1. Elastase and MMP-1 inhibition activity (%) of EEs at 10 mg/mL.
a-d, A-D means with different letters were significantly different ($P<0.05$).

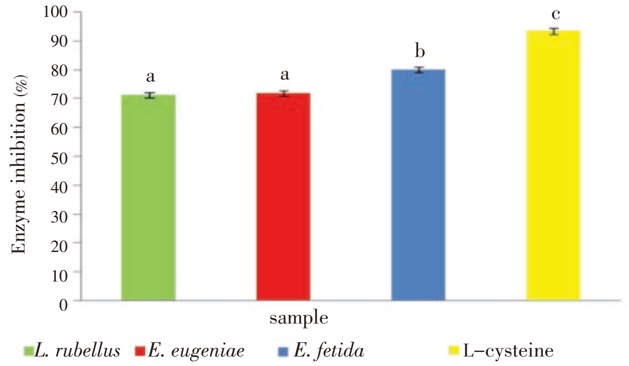

Figure 2. Tyrosinase inhibition activity (%) of EEs and L-cysteine at 0.25 mg/mL.
a-c means different letters indicate that values were significantly different ($P<0.05$).

4. Discussion

The elastic fibers of connective tissues and tendons are mainly constituted of elastin. Under the epidermis, the elastic fibers form a network associated with collagenase fibers. The key enzyme capable of attacking all the major connective tissue matrix protein is elastase. A method that retards elastase activity, such as elastase inhibition assay, could be applied to shield skin from aging, which is easy to the deterioration of elastic fibers by elastase secretion and activation caused by UV light and reactive oxygen species[20]. Our results showed that *L. rubellus* has significantly higher inhibition rate than *E. fetida* and *E. eugeniae*. It is presumed that *L. rubellus* probably contains bioactive peptides that specifically inhibited elastase enzyme regardless of the peptide's molecular weight. There are no data available in the literature to compare the result with regard to elastase inhibition activity of EE. Bioactive peptides have been defined as specific protein fragments that have a positive impact on body functions or conditions and may ultimately influence health[24]. A study reported that tri macrocyclic peptide of porcine pancreatic and human neutrophil elastase inhibitor known as FR901451 was successfully discovered[25]. These preliminary results also suggest that the activity of the elastase inhibitors from earthworms in *E. fetida*, *E. eugeniae* and *L. rubellus* on porcine pancreatic elastases may be helpful in the treatment of inflammation on human leukocyte elastases. Further investigations on the properties of the elastase inhibitor are needed to refine and purify the inhibitor, as well as to determine its relationship to mycosis and its effectiveness against inflammation caused by elastases.The inhibitory activity of MMP-1 on EE was evaluated by MMP-1 colorimetric drug discovery kit (Enzo Life Sciences, United States). MMP-1 is secreted from human skin fibroblasts and is very crucial for inhibitor screening due to its involvement in the degradation of collagen damage from photoaging process in human skin[7]. Basically, it cleaves the X-Gly bond in collagen and Pro-X-Gly-Pro in synthetic peptides where X is an amino acid, resulting in collagen degradation[25]. The ECM degradation process by MMPs proteolytic activity was regulated by their major endogenous protein inhibitors, TIMPs[26]. There are different postulated mechanisms of inhibition or down regulation of MMPs. Inhibition may take place by interaction with an active Zn^{2+} site or by cleaving the active enzyme or binding it to a non-active complex form. The results suggested that earthworms of *E. eugeniae*, *L. rubellus* and *E. fetida* probably contained TIMP-3 which specifically inhibits MMP-1 enzyme[27]. It binds with the enzyme and forms a binary non covalent MMP-TIMP complex, thus blocking the substrate cleavage binding sites[28]. Hence, the EE have the capability to protect collagen degradation where it counteracts with the MMPs by inhibiting its activity thus limiting the breakdown of ECM. This activity of EE may contribute to the reduced degradation of dermal tissue and decreasing the damage level of inflamed or photoaged skin. It is notable that all the extracts possessed activities in both assays. To the best of my knowledge, this is the first report of elastase and MMP-1 inhibitory activities by EE. Nevertheless, most of inhibitory capacity on MMP-1 activity study can be found commonly in plant samples such as white tea, green tea, persimmon leaf, *Sanguisorba officinalis* and flavonoids[5,29-31]. On the other perspective, according to Park, *et al.*[32] peptides especially tyrosine peptides could inhibit tyrosinase enzyme by oxidation of L-3,4-dihydroxyphenylalanine in competitive inhibition. This process enhances the pigmentation decomposition resulting in lighter skin. Thus, the inhibition of EE might be due to tyrosinase-binding in EE which is considered to contain peptides that might be inhibitors of tyrosinase. In addition, there is no published report on anti-tyrosinase activity from earthworms. The mechanism of tyrosinase inhibition from EE was still unknown. Further studies are highly encouraged to optimize and authenticate the tyrosinase-inhibiting activity of potential tyrosinase-inhibiting peptides contained in EE since the preliminary result showed EE has potential use as a cosmeceutical agent in future.

EE of *L. rubellus*, *E. fetida* and *E. euginae* exhibited satisfactory anti-elastase activities ranging from 47% to 53% inhibition and excellent MMP-1 inhibitory activity (72-81%). This study indicated that the EE can be potential candidates for anti-aging cosmetics as well as in other healthcare products where skin evaluation, irritation and eye shall be conducted in future for cosmeceutical application and to be included in finished cosmetics products.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Earthworms extract (EE) has been traditionally used in Chinese medicine for the treatment of various problems from the common disease like flu to the deadly ones such as cancer, heart attack, asthma, and bronchitis. Furthermore, it has anti-inflammatory properties and is used for wound healing in China and other parts of Asia. Therefore, there is need of study to investigate the potential anti-aging activity for EE.

Research frontiers

The present study depicts anti-aging activity of EE assessed by elastase and MMP-1 inhibitory activities and compared with the positive controls.

Related reports

MMP-1 and elastase both are reported cause the degradation of extra cellular matrix (e.g. collagen and elastin) in human skin. The traditional Chinese medicine has evidence of effectiveness of EE in treating skin diseases especially for wound healing and anti-inflammatory agent.

Innovations and breakthroughs

Previous studies on EE have shown its detoxic, antiallergic, antioxidative, antimicrobial, anticancer and anti-inflammatory activities. In the present study, authors have demonstrated the anti-aging activity of EE assessed by inhibition of MMP-1 and elastase activity.

Applications

From the literature survey it has been found that the topical application of EE is safe to humans. This scientific study support and suggest EE has the capability as an anti-ageing agent that is suitable for use in cosmetic products.

Peer review

This is a valuable research work in which authors have demonstrated the anti-ageing activity of EE as ant-ageing agent. The activity was assessed based on the inhibitory effect of EE against elastase and MMP-1 activities.

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