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Effect of Earthworm *(Pheretima posthuma)* Extract as Anti-Oxidant, Anti-Inflammatory and Anti-Angiogenesis

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ABSTRACT

Products derived from organisms play an important role in the discovery of new treatments of different chronic and debilitating diseases. Examining the components of annelids has provided a slew of biologically active substances over the years. Antifungal, antibacterial, anti-allergic, antioxidant, anti-inflammatory, and a variety of other properties medically useful properties were found in annelid components. This study investigated the antioxidant, anti-inflammatory, and anti-angiogenic properties of *Pheretima posthuma* an annelid Asian species. The earthworm's methanol and aqueous extract were tested after it was sacrificed (05 percent ethanol). The anti-angiogenic efficacy of *Pheretima posthuma* was investigated in-vivo utilizing a Chorio Allantoic Membrane (CAM) assay using distilled water extract., the antioxidant capacity of earthworm (*Pheretima posthumous*) was determined using methanol extract and in-vitro antioxidant tests of 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and hydrogen per oxide (H_2O_2). A membrane stabilizing assay and a heat induce hemolysis assay utilizing distilled water extract were used to regulate the anti-inflammatory activity of earthworm hypotonicity (HRBCs). Protein denaturation was inhibited by 59.72 percent in the HRBC membrane stabilizing experiment at 80µg/ml. The aqueous extract of *Pheretima postmortem* exhibits the maximum inhibition at 80µg/ml, i.e., 62.16 percent, in the HRBC membrane stabilizing experiment. The extract was synthesized and tested using an antioxidant DPPH scavenging assay, it produced a great outcome at 40µg/ml, was 54.01 percent. The maximum H_2O_2 scavenging capacity of the *Pheretima posthuma* methanol extract was recorded 69.80 percent at 80µg/ml. The highest anti-angiogenic activity was detected at 80µg/ml, i.e., 62.16 percent using aqueous extract of *Pheretima posthuma*. In conclusion, the earthworm extract caused significant changes and has the capacity to influence angiogenesis-growth of new blood vessels; to prevent and treat neoplastic diseases in human beings.

INTRODUCTION

In Pakistan, the most common earthworm is *Pheretima posthuma.* It's a natural species that has earned a lot of scientific attention. It's an unrecognized and acknowledged champion of soil all over the world. Soil engineers are segmented worms, and Aristotle referred to them as the earth's intestine (Yadav *et al.,* 2017). The ecosystems of earthworm species have been documented by (Saira *et al.,* 2018) in Pakistan. In agricultural areas, meadows land, woodland orchards, and riverbanks, earthworm abundance and burrowing activities were investigated. Earthworm specimens collected in Pakistan revealed 20 species, 12 genera, and 05 families. Lumbricidae (12 percent), Monniligastridae (8 percent), Tubificidae (3.2 percent), Naididae (3.4 percent) and Megascolecidae (73 percent) are the five families. According to Megascolecidae, *P. Hawaii, P. Morris*, and *Pheretima posthuma* are the most productive and widespread species in various specialties (Ashfaque *et al.,* 2018). Bioactive compounds found in marine non-

chordates are also used in ancient medicine.

Bioactive chemicals from non-chordates marine include non-isoprenoid terpenoids, steroids, isoprenoid, and nitrogen-Sulphur heterocyclics brominated compounds, quinones, and nitrogen heterocyclics. Poriferans, annelids, cnidarians, mollusks, echinoderms and arthropods are non-chordates that could be affluent sources of anti-carcinogenic, anti-inflammatory, and antibacterial medicinal compounds (Daimari *et al.,* 2023). Inflammation is a generalized immunological reaction that occurs in response to physical injury of any kind. In some situations, the generally self-limiting inflammatory action becomes chronic, resulting in the onset of chronic inflammatory diseases. Blood flow is improved. Increased cellular metabolism, vasodilation, release of extravasation of fluids soluble mediator, and cellular influx are the cardinal indicators of inflammation that occur in response to any type of physiological injury. In some problems, the self-limiting inflammatory process becomes chronic, and chronic inflammatory diseases occur as a result (Herrero *et al.,* 2022). Uncontrolled inflammatory reactions can lead to the evaluation of acute and chronic inflammatory disorders. Consequently, anti-inflammatory medicines must be used in the immunological response. However, due to the numerous consequences of NSAIDs, such as cardiovascular, renal, and gastrointestinal damage, there has been a rising focus on the usage of natural substances. (Bian *et al.,* 2020).

Inflammation, cancer-related, can be categorized into 2 types. First is that chronic inflammatory conditions cause injuries in tissue, which increases the risk of cancer's risk. (Afify *et al*., 2022). Second, in cancer tissues inflammation is initiated that have elevated after being free of noncancerous inflammation (Packer *et al.,* 2020). Regardless of the cause of the inflammatory ailment, the inflammatory microenvironment is crucial for the preservation and advancement of cancer progression (Klionsky *et al.,* 2021).

Angiogenesis is the process of creating new blood vessels from pre-existing ones or altering the shape and structure of those that already exist (Ansari *et al.,* 2022). Angiogenesis of tumor is required for the initiation and progression of neoplasm. Tumor blood channels, for example, because of morphological differences, have been shown to differ from their regular counterparts. Tumor endothelial cells are genetically normal despite their morphological and functional defects, which is an important principle in tumor angiogenesis (Hida *et al.,* 2018)

Oxidative stress is a situation in which there is instability between antioxidants and free radicals, and where the free radicals' number and amount is greater than the amount and number of antioxidants. A damaged electron in the outermost shell or shells is present in free radical. When there is rise in oxidative stress, it has a deleterious impact on the components of structure cell membrane, such as degradation of lipid, protein, carbohydrate, DNA damage, and malondialdehyde production (MDA), all of which lead to cellular death and hemolysis (Samatra *et al.,* 2017).

Oxidants and free radicals are essential for carcinogenesis during the initial, growth, and promotion stages (Barbaro *et al*., 2020). In the early stages, no fatal mutations accumulate in healthy cells. At an early stage, ROS participates in cell modification by stimulating oxidative damage to DNA and the production of other powerful reactive oxidative products such as lipid peroxides (Poprac *et al.*, 2017). As a result of this free radical's mutagenesis, two key processes play a role in cancer development. The first includes both amplified DNA proliferation and synthesis because of mutation. The second mechanism is caused by an imbalance between apoptosis and cell improvement (Poprac *et al.*, 2017).

MATERIALS AND METHODS

Ethical Approval, Study Design and Area

This research project was duly approved by the ethical review committee. The specimens were collected by spade method from different areas of district Mardan and the experiments were performed in the central research laboratory in the department of Zoology, Abdul Wali Khan University Mardan (AWKUM), Khyber Pakhtunkhwa, Pakistan.

Sample Collection

During the summer, 500 sexually matured and clitellated earthworms were collected. A one-square-foot hole was dug with a spade scraper and collected earthworms were preserved in that hole until they were used and slaughtered using a 5% ethanol solution.

Experimental Design, Chemicals and Drugs

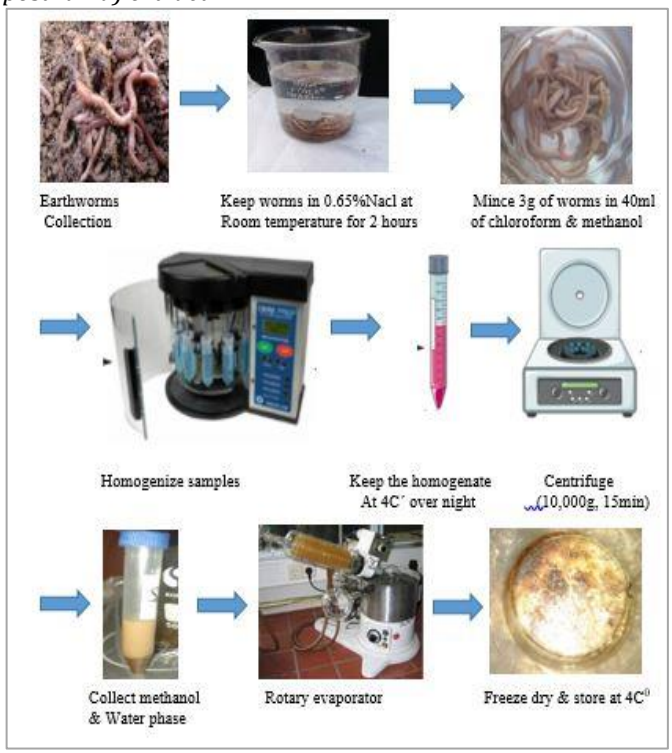
Diclofenac sodium was used as an anti-inflammatory standard, Albendazole for anti-angiogenesis. Vitamin C (ascorbic acid) was used as standard for anti-oxidant. Other chemicals like Chloroform, methanol, ethanol, DPPH, distilled water and H_2O_2 were available during experiment.

Procedure of Earthworm Extract Preparation

Clitella and sexually maturated earthworms averaging 6-8 cm in length were collected from the storage area. The earthworms were cleaned with tap water to detach the sand particles from their bodies. After washing the earthworms, they were submerged in 0.65 percent sodium chloride (NaCl) solution with little changes for 1-2 hours at room temperature until their digestive system was thoroughly cleaned.

The earthworm was then removed from the solution and scarified with a 5% ethanol solution before being minced with scissors. Three gram (3g) of earthworm tissue were taken and homogenized in 40ml of methanol-chloroform (v/v) and the solution was left at 4°C for overnight. Next day, 16ml of distilled water was added to the homogenate solution. After combining the distilled water, it was then filtered through whatman's paper to remove any leftover debris or contaminants and then the samples were centrifuged for 10 minutes at 3000 g/rpm. Three distinct layers were appeared. The water/methanol covering was pipette out and evaporated on a rotary till no methanol were remained and obtained a prismatic and opalescent fluid with a pH of 7. They were then watertight and stored at 4°C until needed. Further, the resulting solution was utilized as a crude extract for various tests (**Figure 01**).

Figure 01
Schematic preparation of earthworm (Pheretima posthuma) extract



Antioxidant Assays performed in-vitro
DPPH scavenging assay by spectrophotometry
The capability of the earthworm extract for scavenging free radicals was tested using DPPH solution (Rehman G., *et al.,* 2018). The *Pheretima posthuma* methanolic extract was utilized to determine the antioxidant activity of earthworms. 1mg DPPH powder was mixed with 25ml methanol to make a 0.1mM DPPH solution. Then, 3 milliliter(ml) of reaction sample was combined with 1 ml of DPPH solution and various concentrations of methanol extract (10, 20, 30, and 40 µg/ml). The percent (%) scavenging of the DPPH radical was determined using the following formula:

$$\text{Scavenging \%} = \frac{\text{Control group} - \text{Sample}}{\text{Absorbance of Control Group}} \times 100$$

Prism graph pad was used and one-way ANOVA was applied to assess antioxidant activity.

H_2O_2 Scavenging Assay in Vitro
According to the potential of earthworm extract to breakdown hydrogen peroxide (H_2O_2) into water and oxygen gas was tested (Mary *et al.,* 2023) with some modifications. A 40mM of H_2O_2 solution was prepared by dissolving 0.6mg H_2O_2 powder in 5ml methanol. This assay used a total reaction mixture of 2ml while 400 liter of H_2O_2 solution was dissolved with various concentrations of extract (20, 40, 60 and 80 µg/ml) and incubated at 37°C for 15 minutes. The absorbance was then measured using a 230nm UV light spectrophotometer.
The results were expressed as percent inhibition.

Anti-inflammatory Assay Performed *in-vitro*
Human RBC- Membrane Stabilization Assay
Human RBC membrane stabilizing method was used for anti-inflammatory activity of earthworms (*Pheretima Posthuma)*. The assay was performed using human red blood cells membrane and their lysis was done through hypotonic saline solution.

Principle of HRBC assay
In this anti-inflammatory assay, against lysis induced by hypo tonicity the membrane stabilization of the extract was checked and the denatured hemoglobin (Hb) content were collected in the form of suspension.

RBCs Suspension Preparation
About 5ml fresh blood was collected from a person who had not taken any steroid drug from 2 weeks and was transferred into Ethylene diamine tetra acetic acid (EDTA) added falcon tube. The blood was centrifuged at 3000 rpm for 15 minutes. The supernatants were discarded and washed with the normal saline solution until become clean and clear. The small rounded mass holding RBCs were secluded and in iso-saline (0.9g of NaCl in 100ml of distilled water) solution resulting 10 % suspension was prepared.

Final Mixture PREPARATION
According to the protocol, following reaction mixtures were prepared.

i. **Control solution (4.5 ml):** This reaction mixture consists of 2ml hypo-saline solution (0.4g NaCl in 100ml of distilled water), 0.5 ml blood suspension, 1ml iso-saline solution and 1 ml PBS.
ii. **Standard solution (4.5 ml):** The standard diclofenac sodium were used as reference and contain 2ml hypo saline and 1 ml various concentration of drugs (10, 20, 30, 40 µg/ml), 0.5 ml blood suspension and 1 ml PBS.
iii. **Tested group:** Aqueous extract of earthworm was added to the reaction mixture in different concentration (10 µl/ml, 20 µl/ml, 30 µl/ml, 40 µl/ml).
iv. **Incubation and centrifugation:** Reaction mixture was incubated at 37C° for 30 minutes and then centrifuged at 3000rpm for 20 minutes.
v. **Spectrophotometry:** After centrifugation, the absorbency was checked through spectrophotometer (UV 5100B) at 560nm and the percent (%) inhibition was checked and recorded.

Heat Induced Hemolysis Assay
The anti-inflammatory assay was also performed by the human red blood cells (HRBC). This assay is based on the concept of stabilizing and lysis of RBC's membrane. High temperature cause lysis of HRBC's. To determine the result, the aqueous extract of earthworm with different concentration (20, 40, 60 and 80 µl/ml) was added into the reaction mixture.

Preparation of RBC's Suspension
The blood was taken from healthy person and was centrifuged at 3000rpm for 10 minutes. When the supernatant become clear it was discarded and the round mass of RBCs were washed with equal volume of PBS. Finally, suspension of 10% v/v in PBS was prepared.

Reaction Mixture

Volume of total reaction mixture was 1ml and the earthworm aqueous extract of different concentration (20, 40, 60 and 80 µl/ml) was added respectively, PBS (860-890 µl), 100µl of erythrocyte suspension (10%) and distilled water were used.

i. **Control group (1ml):** Diclofenac sodium was used as a control. Diclofenac sodium (20, 40, 60 and 80µg) was mixed with PBS (860-890µl) and 100µl of blood suspension (10%).
ii. **Test sample (1ml):** The earthworm aqueous extract was added in different concentration (20 µl/ml, 40 µl/ml, 60 µl/ml and 80 µl/ml) into the reaction mixture.
iii. **Incubation and Centrifugation:** Tested samples were incubated in eppendorf tubes at 54C^0 for 30 minutes. The reaction mixture was then centrifuged at room temperature at 5400rpm for 5 minutes. The absorbency was checked after incubation at 540nm. Percent inhibition of HRBC's lysis were calculated using the same formula and results were recorded.

Anti-angiogenic assay performed *in-vivo*

Chorio Allantoic Membrane (CAM) Assay

In-vivo fertilized chicken eggs were used in this assay and the anti-angiogenic action was tested through the *CAM* assay. Albendazole, phosphate buffer saline and methanol were used. The sterilized eggs obtained from local hatchery were incubated for four (4) days at 37.5°C with absolute humidity (55–60%). On the incubation of 6th day, the extract solutions in PBS of test samples were prepared with different concentrations (40liters/ml, 80liters/ml, 120liters/ml and 160liters/ml) of standard solution i.e., Albendazole (100g/ml). 200 µl of each sample were added to all sets of eggs producing embryos (control, standard and test sample) and incubated for the 24 hours. Chick embryos and *CAM* were photographed on 7th day with a high-megapixel camera. The percent anti-angiogenic activity of all the samples were determined as:

$$\% \text{ Anti-Angiogenicity} = \frac{\text{Control group} - \text{Test sample}}{\text{Control group}} \times 100$$

Statistical Analysis

The data were entered into excel spreadsheet and analyzed through SPSS software 20.1version. The group difference was determined by one-way ANOVA test and the values with *p-value* < 0.05 were considered statistically significant.

RESULTS

Antioxidant Assays activity performed *in-vitro*

DPPH scavenging assay by spectrophotometry

The earthworm concentrations of 10, 20, 30 and 40µg/ml were tested and scavenging rates of 17.41, 27.67, 39.73 and 54.01% were recorded respectively. The greatest DPPH scavenging recorded was 54.01% at 40 µg/ml and the maximum prevention of standard ascorbic acid was 77% at a concentration of 40 µg/ml (**Figure 02**). The results were statistically significant using one-way ANOVA analysis (**Table 01**).

Figure 02

DPPH radical scavenging activity by PPME (Pheretima posthuma methanol extract) Standard Ascorbic acid. All the results are in mean and standard error of mean.

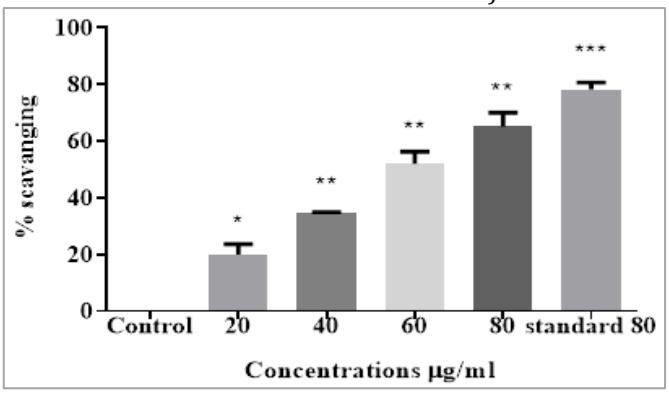


Table 01

Scavenging activity of 2,2-Diphenyl-1-picrylhydrazyl (DPPH).

Group Comparisons (µg/ml)	Mean Difference	95% CI[a] of off	Summary
Control vs 10	-14.04	-27.19 to-0.88	*
Control vs 20	-33.3	-54.46 to-12.13	*
Control vs 30	-44.47	-48.31 to-40.62	***
Control vs 40	-60.93	-67.57 to-54.29	***
Control vs Stand 40	-76.83	-85.54 to-68.11	***

*[a]: Confidence Interval; *, ** = p-value < 0.05 considered statistically significant.*

H_2O_2 spectrophotometry scavenging assay in-vitro

Scavenging activity was observed at concentrations of (20, 40, 60 and 80µg/ml) yielding 21, 34.54, 55.55 and 69.80% respectively. At 80 µg/ml, the maximum scavenging of H_2O_2 was recorded i.e., 55 percent. At a concentration of 80 µg/ml, standard ascorbic acid showed maximal prevention of 80% as shown in **Figure 03**. Results of H_2O_2 scavenging activity along with statistical analysis are shown in **Table 02**.

Figure 03

H_2O_2 radical scavenging activity of PPME (Pheretima posthuma methanolic extract) Standard Ascorbic acid. All the results are in mean and standard error of mean.

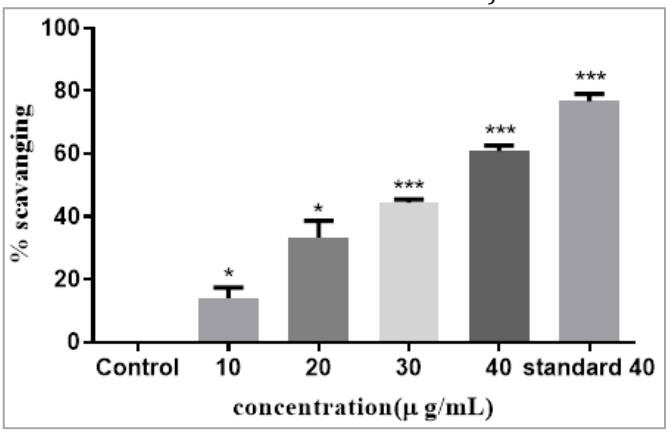


Table 02

H_2O_2 Scavenging activity along with statistical analysis

Group Comparisons (µg/ml)	Mean Difference	95% CI[a] of off	Summary
Control vs 20	-19.99	-34.52 to-5.471	*

Control vs 40	-34.49	-36.24 to 34.74	**
Control vs 60	-52.15	-68.24 to -35.83	**
Control vs 80	-65.16	-84.54 to -45.78	**
Control vs Stand 80	-78.32	-78.65 to 69.00	***

*[a]: Confidence Interval; *, ** = p-value < 0.05 considered statistically significant*

Anti-inflammatory assay results *in-vitro*

Human red blood cells (HRBC) membrane stabilizing assay

Results of different concentration of earthworm extract as well as standard and control were used i.e., concentrations of 20, 40, 60 and 80µg/ml with results of 15.56, 31.03, 41.51 and 62.16% respectively were recorded. Maximum inhibition of 62.16% was observed at 80 µg/ml of extract **(Table 03)**.

Table 03

Results of human red blood cell (HRBC) membrane stabilizing assay

Group Comparisons (µg/ml)	Mean Difference	95% CI[a] of off	Summary
Control vs 20	-15.08	-20.17 to -9.98	*
Control vs 40	-30.04	-40.93 to -19.15	**
Control vs 60	-43.35	-60.16 to -26.53	**
Control vs 80	-60.08	-81.78 to -38.38	**
Control vs Stand 80	-74.66	-104.8 to -44.51	**

*[a]: Confidence Interval; *, ** = p-value < 0.05 considered statistically significant*

Heat Induced Hemolysis (HIH) assay

The results of % inhibition of earthworm aqueous extract was recorded as 15.0, 31.0, 49.0 and 64.5% respectively at a concentration of 20, 40, 60 and 80µg/ml. While 80µg/ml of diclofenac sodium inhibited inflammation by 80% (**Table 04; Figure 04)**.

Table 04

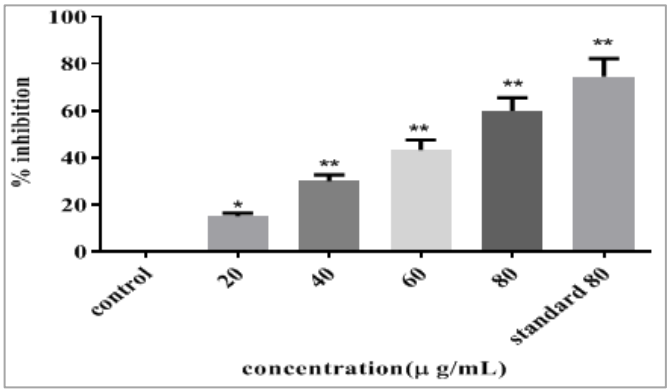
Results of Heat Induced Hemolysis (HIH) assay

Group Comparisons (µg/ml)	Mean Difference	95% CI[a] of off	Summary
Control vs.20	-15.32	-23.4 to -7.144	*
Control vs.40	-28.88	-41.0 to -16.75	**
Control vs.60	-48.26	-56.9 to -39.58	**
Control vs.80	-60.03	-76.5 to -43.52	**
Control vs. stand 80	-76.99	-89.6 to -64.29	**

*[a]: Confidence Interval; *, ** = p-value < 0.05 considered statistically significant*

Figure 04

Results of Heat Induced Hemolysis (HIH) Assay. Comparison of the standard diclofenac sodium (µg/ml) and effectiveness of the extract against inflammation in terms of percent inhibition.



Anti-angiogenic assay performed *in-vivo*

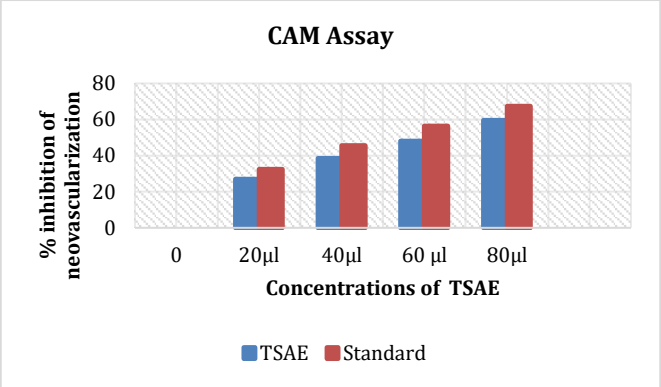
Chorio Allantoic Membrane (CAM) assay

The embryo was exposed and photographed using a high-resolution camera for a more thorough examination on the seventh day after the eggs have been incubated. Adobe Photoshop was used to crop and sharpen the images. In a clockwise manner, the terminal veins were counted. The lower upper area of the CAM and fewer vessels indicated anti-angiogenesis activity. The percent anti-angiogenic activities were recorded.

The maximum extract inhibition measured at 80µl was 60% while the maximum standard result obtained at 80µl was 65.4% (**Figure 05).**

Figure 05

Comparison of Chorio Allantoic Membrane (CAM) Assay



Effect of adult PPE on neovascularization

The *in-vivo* chorio allantoic membrane assay was determined with slightly modified method of Mitrevska *et al.,* 2023. After application of extract doses, the 7th days old embryo was observed for the efficiency of extract on blood vessels.

Diameter, length and CAM Area of vasculatures

The scanning probe image processor (SPIP) software was used and the diameter & length of CAM area of all groups were quantified. Repeatedly thinning in the diameter of primary blood vessels (PBVs) and secondary blood vessels (SBVs) whereas tertiary blood vessels (TBVs) network disappeared with light yellow color appearance.

Results of anti-angiogenic activity of the adult *Pheretima posthuma* extract (PPE) which were dose depended manner, showed that a decrease in diameter of blood vessel occur with the gradual increases in concentration in control group length of PBVs (5.142 nm), SBVs (4.960 nm) and TBVs (3.870 nm) (**Table 05)**. The vasculature length of treated (adult PPE) groups were also found decreased with the increase of concentrations numerical data (**Table 06; Figure 06**).

Table 05

Diameter (nm) of control and treated blood vessels were measured through Scanning probe image processor (SPIP) software. Control group, 20µg(A), 40µg(B), 60µg(C), 80µg(D).

Diameter of Blood Vessels (nm)	Concentrations of adult *Pheretima posthuma* extract (PPE) (µg)				
	Control	A20	B40	C60	D80
PBVs *	1.16	1.402	1.334	1.051	0.972
SBVs**	1.026	1.3	1.122	0.823	0.802
TBVs***	0.839	0.826	0.755	0.741	0.7

Primary Blood Vessels; ** Secondary Blood Vessels; * Tertiary Blood Vessels*

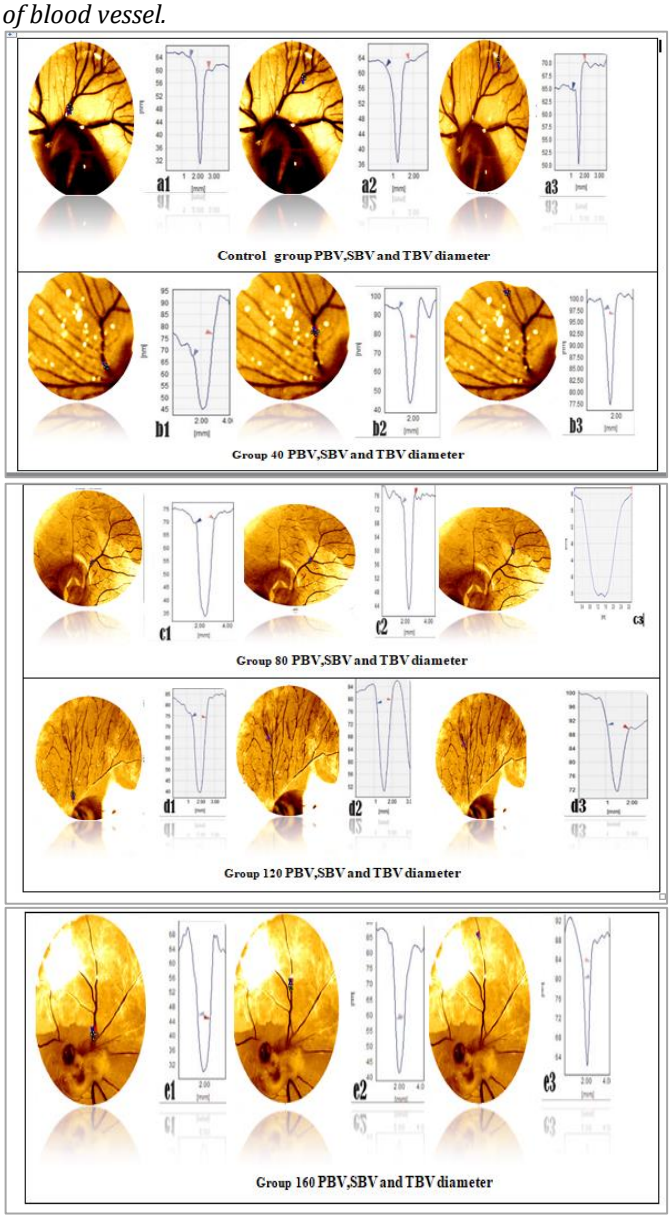
Table 06

Length (nm) of control and treated blood vessels were measured through Scanning probe image processor (SPIP) software. Control group, 20µg(A), 40µg(B), 60µg(C), 80µg(D).

Length of Blood Vessels (nm)	Concentrations of adult *Pheretima posthuma* extract (PPE) (µg)				
	Control	A20	B40	C60	D80
PBVs	5.142	5.09	5	4	2.44
SBVs	4.96	4.03	3.2	3.12	2.1
TBVs	3.87	3.01	2.92	2.701	2

Figure 06

Anti-angiogenic activity of the adult Pheretima posthuma extract which were in dose depended manner, as the concentration increases with gradual decrease in diameter of blood vessel.

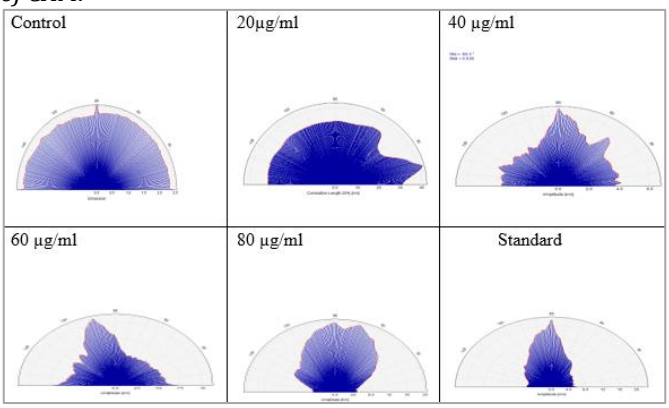


Angular Spectrum representation

Angular spectrum was used and showed the distribution of blood vessels on the whole surface of CAM. Adult *Pheretima posthuma* extract treated CAMs showed inhibition of angular spectrum than untreated control group **(Figure 07).**

Figure 07

Adult Pheretima posthuma extract treated CAMs showed inhibition of angular spectrum than untreated control group and distribution of blood vessels on the whole surface of CAM.



DISCUSSION

Guang-Pheretima is commonly used in traditional Chinese medicine (TCM) for the curing of asthma, temperature, many other inflammatory disorders and it is a "top 10 prestigious Guangdong medicine" (Huang C., *et al.,* 2018). Earthworm extracts are naturally available and have a low risk of causing harm. The concentration of natural extracts that cause cytotoxicity, the type of cell damage, the various stages of cancer cell cycle arrest and the method by which these extracts endeavor anti-cancer effects are currently under research in the world renown research centres. Over the last few decades, researchers have attempted to study alternative therapeutic methods and remedies to prevent cancer progression with minimal success and high failure rates. These concepts have recently evolved to employ naturally available extracts to limit cancer cell division. In the current environment, "Biomolecules against cancer" are a relevant and vital topic since it's critical to uncover biomolecules that can suppress uncontrolled division of cells. Limited research literature is available on the anti-angiogenic and anti-inflammatory properties of *Pheretima posthuma*. The current study was aimed how adult earthworm *Pheretima posthuma* extracts shows anti-inflammatory, anti-angiogenic and anticancer properties.

The first major research into the antibacterial activity of earthworm species was conducted in India and they studied some species of earthworms i.e; *Megascolex konkanensis*, *Lampito mauritii*, *Megascolex, Drawida lennora* & *Drawida impertusa* and utilized their coelomic fluid to discover antibacterial activity for disease-causing microorganisms such as *Vibrio para haemolyticus* and *Bacillus subtillus* which cause disorders such as nausea, diarrhea, vomiting, fever and abdominal pain. Many harmful effects also produced in the human body because of the environmental contamination. Earthworms are good decomposers who aid in the decomposition of decaying waste and lessen the pollution's negative impacts.

Radiation and cancer medications have an aberrant effect on the organism as they cause oxidative stress which activates p53 enzyme activity and induce apoptosis. Mitochondria produce cytochrome upon apoptotic signal to counteract the negative consequences. Cisplatin, anthracyclines, mitomycin and bleomycin with active oxygen are anti-cancer medicines and control the cellular redox status.

The anti-inflammatory, antioxidant and anti-angiogenic properties of adult *Pheretima posthuma* extract were investigated such as for anti-inflammatory assay, a heat induced HRBC denaturation assay maximum inhibition recorded was 64.5 % at 80 µl; membrane stabilizing assay at 40 µl with 53.84 %; and *in-vitro* anti-oxidant activity recorded was 62.50% at 40 µl via DPPH scavenging assay. The maximal H_2O_2 scavenging activity demonstrated by the adult *Pheretima* methanolic extract was 60.86% at 80 µl.

Angiogenesis is a critical phase in the conquering, development and metastasis of solid tumors. Anti-angiogenesis therapy is extremely beneficial in the prevention and treatment of breast cancer. Angiogenesis can be controlled in two ways i.e; indirect and direct mechanisms. The indirect pathway expresses the action of angiogenic proteins that stimulate angiogenesis by affecting the activation of receptors on vascular endothelium. The angiogenic process is thought to play a part in tumor progression, invasion and metastasis and often used as a predictor of prognosis (Fleischer *et al.,* 2023).

In this research study, a chicken egg Chorio Allantoic Membrane (CAM) was used to perform an anti-angiogenic assay. The anti-angiogenic activity recorded of the aqueous extract of *Pheretima* at 80 µl was the highest and was 59.61 %.

CONCLUSION

Certain earthworm species have exceptional characteristics like antioxidant, anti-pyretic, anti-microbial and anti-proliferative potentials as investigated by many researchers throughout the world. However, limited research work has been done in Pakistan on the extract of the earthworm *Pheretima posthuma* to assess its anti-inflammatory, antioxidant and anti-angiogenic capabilities. Many species of *Pheretima posthuma* possess bioactive components.

Earthworms have been used for wound treatment since prehistoric times. Fever, asthma, allergies, bone surgery, heart and brain disorders, and other illnesses were treated using earthworm crude extract.

It is concluded that the potential of adult *Pheretima posthuma* extract having *in-vitro* antioxidant and anti-inflammatory as well as *in-vivo* anti-angiogenesis action (Chorio allantoic membrane assay) were investigated using the chicken egg and prominent results were obtained first time in the study area. All treated blood vessels exhibited a depletion in growth to which the earthworm extract was applied which reflects that adult *Pheretima posthuma* has the potential and capability to influence angiogenesis-growth of new blood vessels; to prevent and treat neoplastic diseases in human beings.

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Authors' Contribution

Tahira Tayyeb: Investigation, Writing-original draft preparation; **Gauhar Rehman:** Supervision; **Asad Ullah:** Conceptualization; **Sulha Syed:** Software; **Muhammad Aslam:** Validation; **Muhammad Owais Khan & Umar Hayat:** Data Curation; **Mansoor Ahmad & Maaz Ahmad:** Resources; **Rafiq Ullah:** Visualization; **Shakirullah Khan:** Methodology; **Fatima Syed & Raheela Taj:** Writing-review and editing; **Shumaila Gul & Rainaz Begum:** Formal analysis; **Imad Khan:** Project administration.

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