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Research Article

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EVALUATION OF ANTIOXIDANT ACITIVITY OF *EISENIA FETIDA*

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Abstract: Antioxidant assay was performed to count the total phenolic content in coelomic fluid of *Eisenia fetida*. The worms were cultured in lab until they became sexually mature. The study focused on total phenolic compound determination using two solvents in a single species of earthworm. It was analysed that the extraction of phenolic compound with 85% ethanol yielded high phenol content, 208.6 mg GAE\L *Eisenia fetida*. Extraction with 85% methanol on the other hand, yielded 189.1 mg GAE\L. *Eisenia fetida* ethanolic extract in the sample manifested high total phenolic content than methanolic extract. Phenols are more readily soluble in ethanol and safe for human consumption. Methanol is used as solvent for low molecular weight polyphenols. The study concludes that ethanol extract of *Eisenia fetida* coelomic fluid contains significant amount of phenols. Phenols have an antioxidant effect and can be used as natural antioxidants for curing ailments related with inflammation and oxidative stress. This study will help in bringing attention towards the therapeutic utility of earthworms.

Keywords: Antioxidant activity, Coelomic fluid, Cold shock treatment, Ethanol, Gallic acid.

INTRODUCTION

Earthworms are significant biotic resources that have an immense potential in agro ecosystems. Earthworms serve as 'soil engineers' to manufacture energy and destroy soil pathogen Sapkota *et al.,* (2020). The earthworm is one of nature's pinnacle 'soil scientists' and acts as secondary decomposer in an ecosystem (Eisenhauer and Eisenhauer (2020). An ecosystem needs a well flourished and balanced biodiversity for sustainability (Prakash and Srivastava, 2019; Verma, 2019). There is a necessity of ecological balance for widespread biodiversity (Verma 2017). The ecological balance is an indispensable need for human survival (Verma 2018). The earthworms' castings have all attributes of ideal manure because they are rich in minerals like magnesium, potassium, nitrates and calcium, phosphorus. Vermicompost contains plant growth regulators, which are responsible for prolific growth of plants (Kiyasudeen *et al.*, 2020). Moreover, naturally available alternatives like azolla have an immense potential of using it as organic compost in fields (Agnihotri, 2019).

Agricultural wastes can be recycled through earthworms, solving many of our alarming issues in respect to water pollution and solid waste disposal (Musyoka *et al*., 2020; Gupta *et al*., 2019). Fellar (2003) emphasized the function and importance of earthworms, by revisiting Darwin's book entitled 'The formation of vegetable mould through the action of worms with observations on their habits' written in 1881. Earthworms sustain the aerobic conditions in the soil. They ingest solids and convert a part of organic matter into worm biomass and respiratory products. They excrete the remaining as castings. Aristotle first noticed their role in turning over the soil and called them 'intestines of earth' (Sinha, 2009). There are about 5000 known species of earthworm on earth (taxo.drilobase.org., 2014; Gabriella *et al*., 2019). The global distribution of earthworm population is under serious influence of climate change and other factors (Phillips *et al*., 2019). For thousands of year earthworms have been used for therapeutics benefits, (Ranganathan *et al*., 2009). They have a fluid filled coelomic cavity that contains watery matrix, the plasma and a large number of coelomocytes. These cells play a very important role in building innate immunity. The coelomic fluid maintains the moisture level and performs physiological activities (Griffith *et al*., 2019). The fluid can be extruded out through the dorsal pores during stress induced by mechanical and chemical agitation (Vučić *et al*., 2019).

Studies have shown that earthworms have unique therapeutic properties, including antioxidative, antitumor antimicrobial and antibacterial (Nagasawa *et al*., 1991; Nakajima *et al*., 2003; Cooper *et al*., 2004; Cooper, 2008; Balamurugan *et al*., 2008; Wang *et al*., 2019; Ding *et al*., 2019). Many scientists have found antioxidant enzyme like sodium dismutase, catalase, and glutathione reductase in the extract of *Eisenia fetida* (Saint–Dains *et al*., 1998). Antioxidants are substances that prevent oxidation, so that normal metabolic functions can be maintained (Ravi Kiran and Aruna, 2010). An oxidation reaction can form free radicals. These free radicals lead to chain reactions that may harm cells. Phenols have antioxidant properties and scavenge the reactive oxygen species to prevent any oxidative damage (Foti, 2007). The estimation of total phenolic content of *Eisenia fetida* helps to establish the use of this species in therapeutics.

In present study, total phenolic content of earthworm extract was measured and significant value (250 mg/L) was found. The Folin- Ciocalteu reagent is used to measure phenol compound of earthworm extract or coelomic fluid. It reacts with the total phenolic content and different amino acids and produces a dark colour that can be measured by spectrophotometer.

The antioxidant activity of phenolic compounds is resolved by their structure, in which a hydrogen atom from an aromatic hydroxyl group is donated to free radicals. This method consists of calibration of a phenolic compound and extraction of phenols from the sample and absorbance are measured after the color reaction. A protocol using the Folin-Ciocalateu was given by Singleton *et al*., (1999). The results are expressed as total phenols mg equivalent to gallic acid /100ml of coelomic fluid. For that gallic acid as standard is used in the assay by Chang *et al*., (2020); Phuyal *et al*., (2020) and Ghafoor *et al*., (2020) confirming that it is the most tested chemical.

Gallic acid is a type of phenolic acid, which is also known as 3,4,5-trihydroxybenzoic acid. It founds in gallnuts sumac, tea leaves, oak and many fruits like pomegranates, grapes etc. $C_6H_2(OH)_3COOH$ is the chemical formula of gallic acid. The gallic acid functions as an antioxidant and defends human cells against oxidative damage.

Pomegranates have strong antioxidant and anti-inflammatory properties (Vučić *et al*., 2019). Pomegranate contains polyphenols as photochemical that preponderate and condensed in the fruit. Pomegranate polyphenols having flavonoid, tannins and hydrolysable tannins are found in peels (rind, husk, and pericarp) (Kharchoufi *et al*., 2018; Singh *et al*., 2018). Pomegranate also contains gallic acid and fatty acids: catechin, epigallocatechin gallate, quertin.

Besides, Exotic Kiwano has anti-oxidant property and is good for wellness, vigour and vitality (Rani *et al.*, 2019).

The *Eisenia fetida* species of earthworm has been recommended by the Organisation for Economic Co-operation and Development (OECD) for testing. Authors carried mostly the standard tests on this earthworm. The characteristic of high reproductive potential and wider adaptability to different environment makes it fit as a model organism. They are endemic to Europe, but have been familiarized (both intentionally and unintentionally) to every other continent except Antarctica. When roughly handled, a red worm exudes a pungent liquid, thus the specific name fetida meaning "foul smelling". This is presumably an antipredator adaptation.

As epigeic worm, *Eisenia fetida* lives and feeds on the soil and form no permanent burrow. These worms are also known as red worms or wrigglers. They have a short life cycle (cocoons hatch within 3-4 weeks) and reach maturity within 7-8 weeks. These worms have a high reproductive output with 2-5 cocoons per week and it is very easy to culture and maintain in laboratory environment (Dom´ınguez *et al.*, 2004). Thus these earthworms have been routinely used in tests for past 50 years. In this study, authors aimed to evaluate the total phenolic content in two different solvents in the red wriggler species.

MATERIALS AND METHODS

Earthworms were collected from Bhoojeevan Organics Private Limited, Delhi for the culture. Earthworms were cultured in labs in pots. To prepare an earthworm bed, coconut peat was placed up to 3-inch depth and levelled. To minimize compaction ground peanut hulls, hardwood sawdust sand or other organic material were mixed into the peat. Small quantity of eggshell and $CaCO_3$ added to adjust pH to about 5.8. Uncontaminated ground water was lightly sprinkled on the bedding. Few adult *Eisenia fetida* worms were introduced into centre of bed. After 1-2 weeks, adult earthworms were collected and exposed to agar medium for gut clearance.

Gut clearance: Before extracting coelomic fluid, earthworm's gut should be clean for preventing any contamination. Agar gel is prepared for their gut clearance. For this purpose, 50ml of 1.5% agar gel is prepared with distilled water. First, this solution is heated until it become transparent. After that the gel was left to cool, and then the gel cut into small pieces. Earthworms were weighed individually and then weighed collectively and then 4-5 earthworms are poured into each jar at constant temperature for 96 hours. The gut was emptied completely after a starvation period of 96 hours. Now again weigh them individually and then collectively and compare their body weight then these were used to coelomic fluid extraction.

Fluid extraction by cold shock method: Some quantities of earthworm (±20gms) were subjected to cold shockby using ice cubes in a Petri plates for about 2 hours and then the fluid is collected in clean dry test tube as used by Sethulakshmi *et al.*, (2018).

Extraction of gallic acid from Pomegranate: Rind was collected from fresh pomegranate and then washed with water to eliminate the impurities. After this, it was dried at room temperature 25°C for few minutes. Then samples were transferred into powder with the help of grinder. Weighed 100g of rind powder and pour in a round bottom flask in 40:60 ratios of with solution content ethanol: water. After that, it was heated in water for 60 minutes at 60°C. Then solution was filtered by filter paper to obtain crude solution (stock solution). The crude solution was distilled to obtain 1/3rd of the solution by using Soxhlet apparatus. Ethanol was refluxed to concentrate crude extraction at 70°C for 3hrs. After that, solution was left overnight at room temperature 25°C to precipitate. Then solution or obtained particles were allowed to dry at 60° in the oven for overnight. Water added in the Soxhlet apparatus and allowed for evaporating at lower temperature.

Antioxidant assay: This is the Calorimetric method which is based on chemical reaction of Folin–Ciocalateu reagent. It is globally used for the quantification of total phenolic content in coelomic fluid of earthworm. This method possesses a calibration with a pure phenolic compound and their extracted from our sample (coelomic fluid) and absorbance or optical density (O.D.) was recorded after the colour reaction. A typical protocol using the Folin-Ciocalateu (FC) was given by Singleton *et al*., (1999). The results were expressed as total phenols mg equivalent to gallic acid per gram of sample. The experiment was done in triplicate.

Sample preparation:

(a) 1ml of sample was extracted from earthworms placed in agar medium in different groups and then mixed with 5ml of 85% methanol in one test tube and 5ml of 85% of ethanol in other test tube.

(b) 200µl of FC reagent was added in each test tube.

(c) After 3 min, 1000µl of 7% Na_2CO_3 was pour into the mixture in each test tube.

(d) The sample was incubated for 2 hours at 25°C room temperature and shaken until homogenized.

(e) **Blank:** 200µl FC reagent was taken. The content was cyclo mixed for 3 minutes followed by addition of 1000µl of sodium carbonate and then treated same as sample.

(f) **Standard:** Standard series of known concentration of Gallic acid (10, 20, 30, 40, and 50mg/l) and then add 200 µl FC reagent and thereafter treated in same way as sample (table 1).

Quantification of phenolic compounds contents:

(a) The reading of (O.D.) absorbance at wavelength 765 nm was recorded by spectrophotometer. The total phenolic content was demonstrated as mg GAE per gm of sample. Calculation of phenolic content is done by applying the formula:

$$T = C \times V \backslash M$$

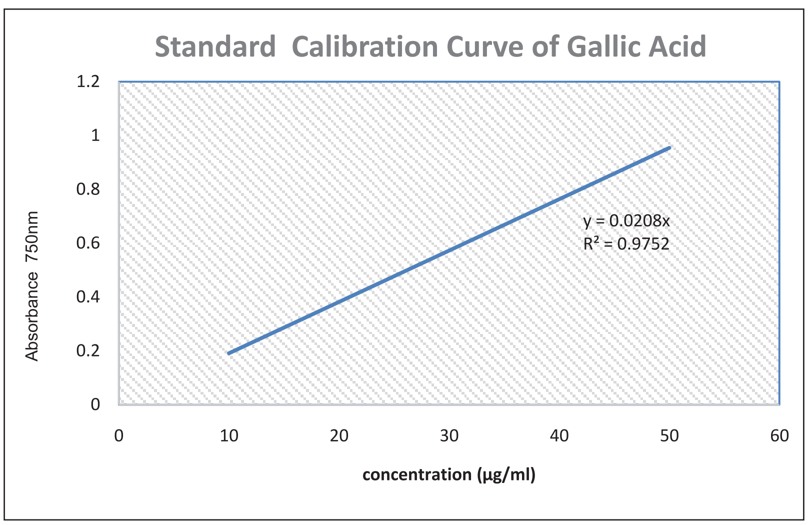
(b) T→ Total phenolic contents in $mg.g^{-1}$ of the extracts as GAE.

(c) C → Concentration of Gallic acid established from the Calibration curve in $mg.ml^{-1}$.

(d) V → Volume of the extract solution in ml and M is the weight of the extract in g.

Table 1: The reading of (O.D.) absorbance at wavelength 765 nm was recorded by spectrophotometer.

Concentration (µg/ml)	Absorbance λ max = 765 nm
10	0.23
20	0.44
30	0.65
40	0.76
50	0.88



Graph 1: Standard calibration curve of Gallic acid.

RESULTS AND DISCUSSION

Authors found statictically significant difference. The p value was less than 0.05, giving strong evidence that different solvents yield difference in the amount of total phenolic content. The descriptive statistic was applied and result were experessed as Mean ± Standard deviation. It was found that the extraction of phenolic compound with 85% ethanol yielded high phenol content, 208.6 mg GAE\Lin *Eisenia fetida*. Extraction with 85% methanol yielded 189.1 mg GAE\L (table 2). *Eisenia fetida* ethanol extract in our sample manifested high total phenolic content than methanol extract. Phenols are more readily soluble in ethanol and safe for human consumption. Methanol is used as solvent for low molecular weight polyphenols. The gallic acid solution of concentration (10-50 μg/ml) confirmed to Beer's Law at 750 nm with a regression co-efficient (R2) = 0.9752 n=3. The plot has a slope (m) = 0.0208 (graph 1). The equation of standard curve is y=0.0208x

Table 2: Showing the result for the total phenolic content.

S.No.	Sample	Total Phenolic content (mgGAE/g) Mean ± S.D.
1.	Methanol	189.1 ± 1.429452109
2.	Ethanol extract	208.6± 0.964365076

Ethanol as solvent extracted phenolic content from the coelomic fluid of the *Eisenia fetida* was significant. The highest concentration (Aldarraji *et al.*, 2013) was taken for coelomic fluid total phenolic content and findings from the yields justify the work done by many scientists (Pinelo *et al.*, 2004; Spigno *et al.*, 2007; Turkmen *et al.*, 2007). Solubility of phenolic compounds were found higher in ethanol. Authors used an ethanol (85%) to macerate our sample .

Authors found high phenol content than methanol in ethanolic extract studied from earthworm samples. Phenols were found more readily soluble in ethanol and safe for human consumption. Methanol was used as solvent for low molecular weight polyphenols (Kapasakalidis *et al.*, 2006). But methanol has been proved to be more productive for some other source of phenolic content like peanut hulls (Yen *et al.*, 1993); cinnamon extracts (Mancini-Filho *et al.*, 1998); peanut skin (Nepote *et al.*, 2005); grapeskin (Cvjetko Bubalo *et al.*, 2016).

This result can be affected due to biology of the worm and different protocols used during extraction. Besides concentration of methanol in water, there are various circumstances which affect the extraction, like size of particles ,chemicals used and methods which are followed during extraction. Factors which effect the biology of earthworms may be physical ,biological and chemical properties of soil,type of food, organic matter and type of microbes and nematodes. Earthworms have a tendency to acquire and concentrate the organic pollutants or inorganic elements. From above observation, authors found that coelomic fluid of earthworm can be an aptitude source of the phenols specifically using ethanol as solvent.

CONCLUSION

This study emphasized the importance of ethanol as solvent in extracting total phenolic content in *Eisenia fetida*. The status of earthworms as 'farmers' friend' can be shared with the status of earthworm's utility to 'doctor's friend'. This experiment further opens the doors for comparative studies on evaluation of total phenolic content of different species of earthworm in different concentrations and types of solvents. The study analysed that the ethanol extract of *Eisenia fetida* coelomic fluid contained significant amount of phenols. Phenols have an antioxidant effect and can be used as a natural antioxidant for curing ailments related to inflammation and oxidative stress.

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