Research Article

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EVALUATION OF EARTHWORM POWDER (*EUDRILUS EUGENIAE*) AND ITS APPLICATION IN COTTON CREPE BANDAGE

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Abstract: From time immemorial earthworms have been used as therapeutic agents. Earthworm possess rich natural sources of antioxidants and are used with other herbs to treat a wide variety of conditions ranging from spasms and convulsions to pain relief, treatment of fever and certain types of arthritis. Focusing this, in our present study, the earthworm powder was prepared and its glucose, protein, lipid, phenol contents were analyzed. The minimum inhibitory concentration and antimicrobial assays were performed against selected human pathogens. The powder was subjected to antioxidant assays. The protein bands were visualized by performing SDS PAGE. The thin layer chromatography was performed to analyze the bio- constituents present in it. The powder which is having the antimicrobial and antioxidant activity was impregnated to cotton crepe bandage to bring out its therapeutic application in bandage.

Keywords: Antimicrobial Assay, Earthworm, Pathogens, Thin Layer Chromatography.

INTRODUCTION

Earthworms have been used in medicine for various remedies since 1340 AD (Stephenson, 1930). Earthworms are the largest members in the Oligochaeta phylum Annelida or segmented worms, terrestrial relatives of certain marine species, and medicinal leeches that are also of clinical relevance. Earthworms also play essential biological, chemical, and physical roles in ecology. According to Darwin, "it may be doubted if there are any other animals which have played such an important role in the world as these lowly organized characters." However, earthworms surprised researchers through their diverse functions beyond improving soil fertility. For example, their behavior and more recently their impressive innate immune potential have captured a new research audience.

Earthworms have been viewed as an important protein source. Sun *et al.,* 1997, confirmed high protein content consisting of 78-79 grams of free amino acids per liter. There is a high content of vitamins and minerals particularly iron (Fe) and calcium (Ca). Paoletti *et al.,* 2003, analyzed earthworm's potential as a source of protein, nutrients and fatty acids for human consumption. The antioxidants in earthworm are also of great interest as protective agents to help the human body reduce the oxidative damage without any interference. Earthworm has been recognized in oriental medicine as anti- inflammatory, analgesic and antipyretic agent. It shows anticancer effect by preventing excess glucose uptake. Microorganisms are known to play a major role in soil characteristics, invertebrates are believed to act as regulators of antimicrobial activity. Earthworm surface excreta were found to have potent antimicrobial activity. There are many earthworm studies that seem to be not related to either Ayurveda or TCM based on antibacterial agents and prophylactic molecules that now have wider implications. Cooper *et al.,* 2004 discovered that earthworms have been prominent with respect to lysis of bacteria and with other implications to disease. Anti-inflammatory activity and antimicrobial potency (Shobha and Kale, 2007) of *Eudrilus eugeniae* of earthworm extracts on certain plant pathogens were studied. Antitumor activities of earthworm fibrinolytic enzyme on human hepatoma cells were studied (Hong, 2007). The anti-inflammatory and antipyretic activities of biologically active extract isolated form whole earthworm, *Lampito mauritii* were determined (Balamurugan *et al.,* 2008).

The various solvent extracts of an earthworm, *Eudrilus eugeniae* were prepared and anti-inflammatory activity of these extracts were determined. The petroleum ether fraction possessed maximum anti-inflammatory activity in carrageenan induced albino rats in comparison to 95% ethanol and 0.2 M phosphate buffer (pH, 7.0) extracts (Abishek *et al.,* 2010). *Eudrilus eugeniae* (Kinberg) is the commonly type of earthworms used for vermicomposting in tropical and sub-tropical countries. The earthworm species, *E. eugeniae* is indigenous to Africa but has also been bred in the USA, Canada, Europe and Asia, where it is commonly called the African night crawler, to be used as fish bait. It grows well at a temperature of more than 25°C but best at 30°C attaining maximum weight,

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length and number of segments in about 15 to 20 weeks. Thus the species selected for study was *Eudrilus eugeniae* which is native of Africa and is having good reproduction and maturation capability.

MATERIALS AND METHODS

Earthworm Culture:

E. eugeniae was collected from the Vermi culturing Unit maintained by the Department of Microbiology, Nehru Arts and Science College, Coimbatore. The earthworm was brought to the laboratory and mass cultured in culture tank (1m×1m × 1 m) containing urine free cow dung. Cow dung was collected from the nearby cattle shed, sun dried and powdered. The worms were acclimatized in cow dung and used for various experimental studies.

Preparation of Earthworm Powder:

The earthworm powder was obtained following the method of Ishii *et al.,* 1992 with slight modification. About 10 gm of sexually mature, clitellated worms (900 mg/worm) were washed with running tap water to remove any dirt from the body surface. The earthworm were fed with tissue paper, thus the alimentary canal of the living earthworm is substantially freed of soil by their own excretory power. The living earthworms are left there at a temperature of 25°C for a period of 72 hours. Thereafter the living earthworms are ground in homogenizer, dried and stored for further use.

Quantitative Estimation of Bioactive Compounds:

Estimation of proteins: About 0.5ml of earthworm powder dissolved in distilled water was taken. The volume was made up to 1ml with 0.1N sodium hydroxide. 5ml of reagent (alkaline copper solution) were added. The content of tube were mixed well and allowed to stand for 10 minutes at room temperature. Then 0.5 ml of reagent sodium tartarate solution was added. It was mixed well and incubated at room temperature in dark for 30 min. Blue colour indicates the presence of proteins. The reading was taken at 660 nm and Bovine serum albumin was taken as the standard (Lowry *et al.,* 1951).

Estimation of sugars: The total sugar content was estimated by phenol sulphuric acid method (Dubois *et al.,* 1956). To the sample about 1 ml of phenol and 5 ml of sulphuric acid were added. The reading was taken at 490 nm and glucose was taken as the standard.

Estimation of Lipid: The lipid content was measured by using the phosphovanillin method and the reading was taken at 520 nm. The cholesterol was taken as the standard (Barnes and Blackstock, 1973).

Estimation of Total Phenols: Total phenols were determined by using the Folin Ciocalteu reagent (Singleton and Rossi, 1965). The sample (0.5 ml) was mixed with the Folin Ciocalteu reagent (5ml of the reagent diluted tenfold with distilled water) and aqueous sodium carbonate (4 ml, 1 M). The mixture was allowed to stand for 15 minutes and total phenol was determined at 765 nm with a double beam UV/visible spectrophotometer.

Estimation of Total Flavonoid: The powder was (0.5ml) mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminium chloride, 0.1 M potassium acetate and 2.8 ml of distilled water. After keeping the mixture at room temperature for 30 min, the absorbance of the reaction was measured at 415 nm with a double beam UV/visible spectrophotometer (Chang *et al.,* 2002).

Anti-Oxidant Activity:

Total Anti-oxidant Capacity: The total antioxidant was measured according to spectrophotometric method of Shirwaikar *et al.,* 2006. Each extract (10mg/ml) to a volume of 0.1 ml were dissolved in water and taken in an eppendorf tube with 1ml of reagent solution (0.6 M Sulphuric acid, 2.8mM Sodium phosphate and 4mM Ammonium molybdate). The tubes were capped and incubated in thermal block at 95°C for 90 minutes. After cooling to room temperature, the absorbances of the solutions were measured at 665nm against blank with a double beam UV/visible spectrophotometer.

Hydrogen Peroxide Scavenging Activity: The hydrogen peroxide scavenging assay was carried out following the procedure of Ruch *et al.,* 1998. The principle of this method is that there is a decrease in absorbance of H_2O_2 upon oxidation of H_2O_2. A solution of 4Mm H_2O_2 was prepared in 0.1M Phosphate buffer (pH 7.4). Various concentrations of extracts (200µg /ml -1000µg /ml) in 3.4ml phosphate buffer were added to 0.6ml of H_2O_2 solution (4Mm) and absorbance of the reaction mixture was recorded at 230nm. A blank solution contained phosphate buffer without the extract.

$$H_2O_2 \text{ Scavenging activity} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}}$$

Antimicrobial Assay:

Test Pathogens: The bacterial cultures used in this study were obtained from PSG College of Arts and Science, Coimbatore. The cultures used were *Bacillus* sp., *Enterococcus* sp., *E. coli*, *Klebsiella* sp., *Proteus* sp., *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The isolates were sub cultured and stored at 4°C.

Minimum Inhibitory Concentration Method*:* Nutrient broth was prepared in test tubes and then sterilized. 24 hours incubated fresh broth culture of *Bacillus* sp., *Enterococcus* sp., *E. coli*, *Klebsiella* sp, *Proteus* sp., *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Then equal amount of culture was added to each test tube. After that extract of

different concentrations (100µl, 150µl and 200 µl, 250 µl, 300 µl) was added to each test tube and incubated for 24hrs. After 24 hours of incubation turbidity of the broth was measured.

Screening for Antibacterial Activity (Agar-Well Diffusion Method) (Perez *et al.*, 1990): The antibacterial activities of the extracts were tested against the selected bacterial strains. Sterile Muller hinton agar plates were prepared and lawn cultures of the organisms were spread on each plate. The wells of 6mm size were cut in the agar plates with the help of sterile cork borer and the wells were loaded with different extracts of various concentrations (100µl, 150µl and 200µl, 250µl, 300µl). The positive (Ampicillin) and negative controls were also used. All the plates were incubated at 37°C for 24-48 hours. After incubation, the plates were observed for the formation of zone of inhibition and the zone sizes were measured.

Thin Layer Chromatography: *TLC* glass plates of size 20 x 20 cm were washed in clean water and kept for drying. Silica gel G (Hi Media) of 60 to 120 meshes was used as stationary phase. The prepared plates were activated by heating at 110 °C for half an hour in hot air oven. The TLC was done to detect the presence of sugars, lipids and proteins in the sample. The plates were spotted with 10 µL extracts using capillary tubes. The solvent phase used were acetic acid: water (15:85), chloroform: methanol: water (65:25:4) and butanol: acetic acid: water (4:3:5). For visualization uv fluorescence for sugars, iodine vapour and Folin-ciocIteau reagents respectively for lipid and proteins were used as the spraying agent.

Detection Of Protein Bands: The crude sample was subjected to SDS PAGE for the detection of various protein bands. The sample was subjected to SDS-PAGE using 12.0% separating gel (Laemmli,1970). After electrophoresis, the bands were visualized by Coomassie Brilliant Blue R-250 staining.

Impregnation of Earthworm Powder on Cotton Crepe Bandage: Cotton fabric was washed, sterilized and dried before use. Experiments were performed on fabric with dimensions of 2.5 × 2.5 cm. In order to impregnate the cotton fabric, it was submerged in conical flask containing the earthworm powder extract and was shaken at 600 rpm for 24 hours. Then it was dried at 70°C for few minutes in hot air oven and kept for further analysis.

Antibacterial activity of Treated cotton crepe bandage cloth: The antibacterial activity of treated cotton crepe bandage was checked against pyogenic organisms, *Staphylococcus aureus, Bacillus* sp, *and Pseudomonas aeruginosa.* Sterile MHA plates were prepared and lawn cultures of organisms were spread on each plate. Controls were kept for each organism. The plates were then incubated for 24 hours at 37° C and were observed for the zone of inhibition.

RESULTS

The earthworm powder (Fig.1) was tested for the presence of protein, carbohydrate and lipid content which was found to be about 65%, 19% and 16% respectively. The phenol content was found to be 250µg/g and 380µg/g of flavonoid content was detected in the sample. The antioxidant activities were found to be increasing with the increasing concentrations of the earthworm powder extract in both total antioxidant activity and hydrogen peroxide scavenging activity (Chart 1 and 2).

Figure.1: Earthworm powder

Chart.1: Total antioxidant activity of the dry earthworm powder.

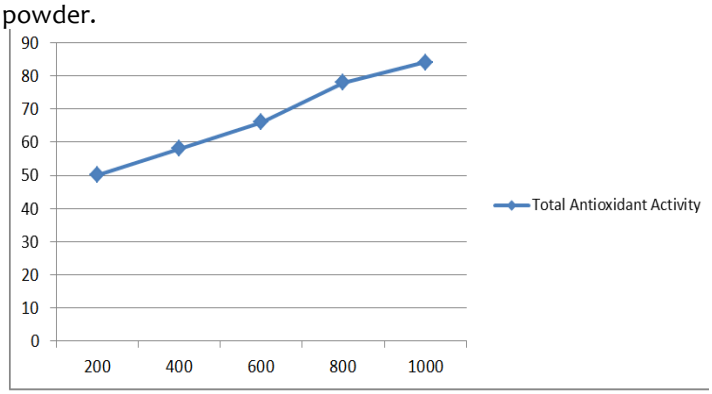
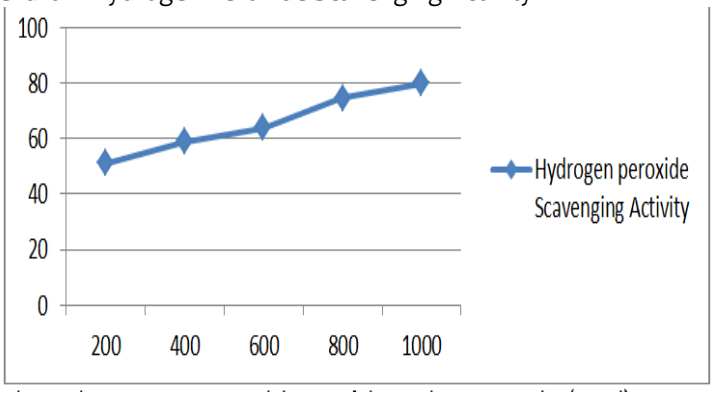


Chart.2: Hydrogen Peroxide Scavenging Activity



The antimicrobial assay was performed against *Bacillus* sp., *Enterococcus* sp., *E. coli*, *Klebsiella* sp, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The minimum inhibitory assay was performed where the percentage inhibition was found to be 45%, 56%, 52%, 90%, 85% and 80% respectively (Chart 3). The disc diffusion test was done where the zone of inhibition were measured in all the selected pathogens. The maximum zone was found to be in *Klebsiella* sp followed by *Pseudomonas* sp. and *S. aureus* (Chart 4, Fig. 2).

Figure.2: Antibacterial activity of earthworm powder

Chart.3: Showing percentage inhibition by the earthworm powder (300µl)

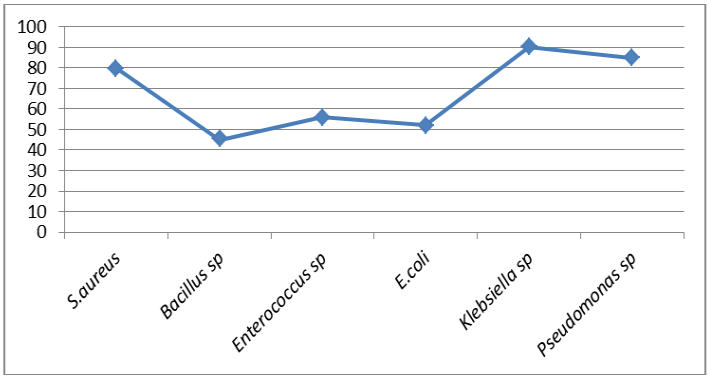
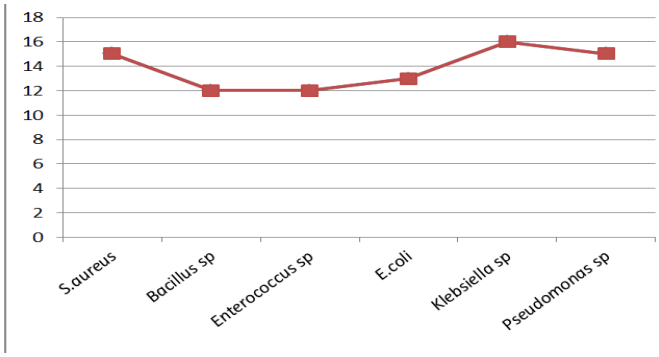


Chart.4: Showing zone of inhibition (mm) of the powder by well diffusion method



The presence of protein, carbohydrates and lipid were detected by TLC. The purple colour spots were found after spraying ninhydrin confirming the presence of aminoacids (Fig.3). The major essential amino acids (proline, arginine, cysteine, hydroxyproline, serine, histidine, phenylalanine, methionine, threonine, alanine, isoleucine and leucine) were detected. The carbohydrates (glucose and mannose) presence was confirmed by the spots formed after viewing the fluoresce under uv lamp. The brown spots were formed after viewing the plates in iodine chamber in case of lipids (fatty acids). (Fig.4). About 8 prominent bands of 10 - 200 kd were observed after performing the SDS- PAGE.

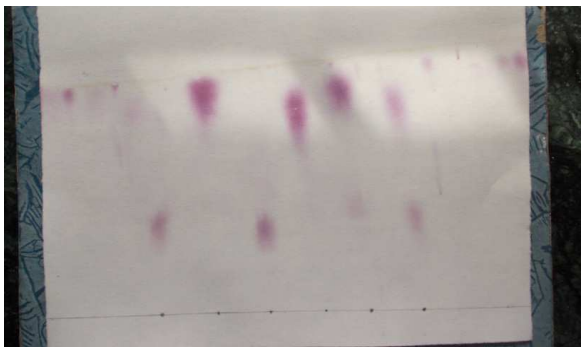


Figure.3: TLC for proteins

Figure.4: TLC of Lipids

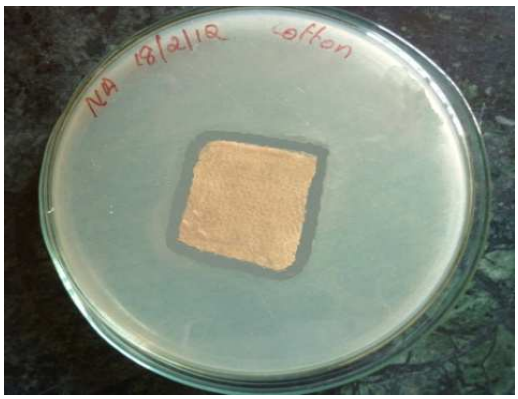


Figure.5: Antibacterial activity of treated cotton crepe bandage cloth

The application of the earthworm extract was applied to the cotton crepe bandage cloth as an *in vitro* test against microorganisms. The organisms having inhibition in antibacterial assays were subjected to this

test. The zone of inhibition was found against *S aureus, Pseudomonas* sp and *Klebsiella* sp (Fig. 5).

DISCUSSION

Earthworms have been widely used in traditional Chinese medicine for a long time. However, in the past few decades with the development of biochemical technologies, the research on the pharmaceutical effects of earthworms has been studied for its use in veterinary and medical field. The biochemical analysis showed the presence of high content of protein and comparable amount of carbohydrate and lipid content. It was earlier reported that earthworm meal *L. rubellus* had high protein content and it was very important as nutrient source for animal. Protein content of earthworm meal was equal to fish meal (55-60% crude protein) as protein source for poultry feed. It has dense nutritional content because of their soil based origin (Istiqomah, 2009). Extracting medicinal compounds from the earthworm has traditionally been practiced by indigenous people throughout the world, more particularly in Asia. Previous earthworm studies have shown its antimicrobial (Cooper *et al.,* 2008), hepatoprotective (Balamurugan *et al.,* 2008), anticancer (Cooper *et al.,* 2004) and scar wound healing characteristics.

The anti-inflammatory activity together with antioxidant properties seems to be due to the high polyphenolic content in earthworm tissue (Balamurugan *et al.,* 2008). In our study the presence of phenol and flavanoid content had also been reported. The antioxidants are of great interest as possible protective agents to help the human body to reduce the oxidative damage without any interference. The antimicrobial assays were also performed and the powder at 300 µg/ml concentration showed inhibition against *S. aureus, Pseudomonas* sp and *Klebsiella* sp. Earlier studies reported that the petroleum ether extract showed maximum potency against *Staphylococcus aureus* in comparison to *Streptococcus pyogens*. Petroleum ether extract was found to possess antifungal activity against *Aspergillus niger* in comparison to *Candida albicans*. Against *E. coli*, ethanolic and petroleum ether extract possessed least antibacterial activity (Abishek Mathur *et al.,* 2010). The antimicrobial activity of the extract in the cotton crepe bandage against selected pathogens had also been studied. The *in vitro* study of impregnation of earthworm powder in the cotton crepe bandage would help in the development of bio- band aid as similar to herbal bandages in the current scenario. Thus implementation of these compounds into the treatment of human and animal diseases as well would help us to be set a goal that should be strived to achieve in the field of medicine.

CONCLUSION

The scientific approach should be able to give solid evidence supportable for the use of the earthworm powder which had already been long-exploited in the folk medicine settings for its application as pharmaceutics. A new approach could be exercised both in comparative and alternative medicine settings with the use of a natural product derived from animal or plant sources. The earthworm powder having high protein content along with the antimicrobial and antioxidant could be used as a food or feed source may also have pharmaceutical applications.

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