SUMMARY

Earthworms are one of the well known beneficial creatures of animal kingdom. They constitute an integral component soil ecosystem as they play an important role in maintaining fertility of the soil through their activities. Their importance in agriculture was highly respected in the past, hence they earned the designation of "Farmers Friend", "intestines of the earth", "soil engineers" etc. With introduction of modern system of agriculture with prolific use of chemical fertilizers and pesticides and mechanical tools, the importance of waste recycling in the soil through microorganisms and earthworms got declined. Now the drawbacks of modern system of agriculture have been recognized and emphasis has been laid of nature friendly methods for sustainable development. In recent past simple method of bio-conversion of organic waste into high quality vermicompost has been developed by using high efficiency epigeic earthworms. The practice of vermicomposting is gradually becoming popular.

Earthworms are essential component of the food chain and energy cycle and are eaten up by a number of animals including invertebrates, fishes, amphibians, reptiles, birds and mammals. Food and medicinal value of earthworms has been recognized since thousands of years and this aspect has been cited in ancient literature of various cultures.

Earthworms have traditionally been used as food and medicine especially of tribal community over the globe. Medicinal properties, particularly for cardiovascular problems, of earthworms have received special attention in Chinese system of medicine. Pioneering research work has been done in China followed by researchers of other countries which have resulted in successful development of an earthworm based drug named as lumbrokinase. Most of the investigations on pharmaceutical properties of earthworms have been done on *Lumbricus rubellus* and few on other species of worms. The present study has been carried out to demonstrate medicinal properties of vermicomposting earthworm *Eudrilus eugeniae*.

A stock culture of earthworms, *Eudrilus eugeniae* was maintained in Vermicomposting Center, located in Charak Udyan, Jiwaji University, Gwalior, (M. P.), India.
extract (samples) was prepared using adult worms. Some of the investigations were done using *in vitro* cell free system and for other work *in vivo* experiments were conducted using male albino rats.

6.1 *In vitro* antioxidant activity:

*In vitro* antioxidant activity of earthworm extract was determined using three methods. A concentration dependent inhibition of DPPH radicals was observed with maximum activity (89.24 ± 0.005) at 4 mg/ml dose followed by other concentration tested, 3 mg/ml (79.24%), 2 mg/ml (65.48%) and 1 mg/ml (54.13%). At still higher concentration, 5 mg/ml (89.11%), a rather lower, and statistically at par, inhibition was noticed than the preceding concentration. A similar, but stronger DPPH radical inhibition was obtained with much lower concentrations of vitamin C.

The second marking parameter of antioxidant activity was reducing power of the samples. The maximum reducing power (0.724±0.0070) was obtained with 5 mg/ml followed by lower concentrations, 4 mg/ml (0.615±0.0035), 3 mg/ml (0.489±0.0115), 2 mg/ml (0.384±0.0005) and 1 mg/ml (0.260±0.0030). The well known standard antioxidant vitamin C exhibited similar dose dependent but stronger results.

The antioxidant activity of some of the substances is also attributed to their higher total phenolic content that can be easily determined spectrophotometrically using tannic acid as standard. The total phenolic content of earthworm extract was calculated to be 25 µg/mg or 250 mg/L that was indicative of its significant antioxidant activity.

6.2 Anti-inflammatory activity:

Acute anti-inflammatory activity of EW extract was demonstrated by monitoring status of carrageenan induced paw oedema in control and treated rats for a duration of 6 hours. It was observed that treatment with different doses of earthworm extract provided significant dose dependent inhibition of rat paw oedema. The results on rats treated with 250 mg/kg of earthworm extract were similar to 10 mg/kg of indomethacin (standard anti-inflammatory drug) treatment. The volume of paw oedema, in both the cases,
increased during first two hours and then decreased to normal volume during 6 hour regimen. The decline in paw oedema is an indication of anti-inflammatory activity.

One more method was employed for demonstration of anti-inflammatory activity *i.e.*, cotton pellet induced granuloma pouch method (chronic anti-inflammatory test). Sterilized cotton pellet (20 mg), impregnated with carrageenan, was inserted in the body of rats and on 8th day wet cotton pellet were carefully removed, dried and weighed to determine increase in their weight due to formation of granuloma pouch, as parameter of degree of inflammation. It was noticed that the weight of excised cotton pad was higher in all 6 groups of rats than its initial weight (20 mg); the maximum increase was found in control groups, indicating maximum inflammation. In indomethacin treated rats the weight of excised cotton pellets was only marginally higher than the initial weight and this was due to already proved anti-inflammatory activity of this standard drug. The treatment of earthworm extracts exhibited dose dependent influence. The lower dose (150 mg) earthworm extract showed higher weight (62.5±5.49) of cotton pellet than the other doses 200 mg and 250 mg showing lower weight gains 54.5±2.72 and 51±4.12 mg respectively due to lower to higher anti-inflammatory activity. Thus, it can be concluded that oral administration of earthworm extract has significant anti-inflammatory activity, but still the effect was inferior to that of the standard drug indomethacin.

6.3 Oxidative stress markers:

The blood samples of experimental rats used for acute and chronic anti-inflammatory tests were used for quantitative measurement of status of oxidative stress markers of antioxidant activity including reduced glutathion (GSH), superoxide dismutase (SOD), thiobarbituric acid reactive substance (TBARS) and catalase (CAT). The lower levels of TBARS are indicative of higher antioxidant activity, while higher levels of other three parameters were indicative of higher antioxidant activity. The results of these antioxidant activity biochemical parameters obtained from blood samples of rats of both acute and chronic anti-inflammatory tests, it was revealed that treatment of rats with earthworm extract and indomethacin provided considerable antioxidative protection. However, clear dose dependent influence was not demonstrated.
6.4 Cytokines in anti-inflammatory activities:

The blood of these experimental rats was also used for determination of cytokines as marker of inflammatory and anti-inflammatory conditions. Declining levels of TNF-α and increasing trend of IL-10 are indications of anti-inflammatory effect of the test material. The findings of anti-inflammatory parameters of cytokines (lower values of TNF-α and higher values of IL-10) in earthworm extract and indomethacin treated rats of both (acute and chronic) experimental groups, provided evidence of considerable anti-inflammatory activity.

6.5 Anti-pyretic activity:

Experimental rats were also used for demonstration of anti-pyretic activity of earthworm extract. It was noticed that treatment of fever-induced (pyrexia) rats with paracetamol resulted in reduction of temperature to normal level during experimental period of 4 hours. Treatment of animals with earthworm extracts also provided similar anti-pyretic effect in a dose dependent manner.

6.6 Antimicrobial activity:

The antibacterial activity of EW extract was determined on selected bacterial isolates on agar plates using well diffusion method. Out of eight bacterial isolates only four responded to earthworm extract. Maximum antibacterial activity 29 ±1 mm was found with gram negative *Pseudomonas aeruginosa*, which was almost similar (29.5±0.5 mm) to the standard antibiotic Streptomycin. Minimum antibacterial activity (21.5±1.5 mm) was obtained against gram positive *Staphylococcus aureus*. Isolates of *Proteus mirabilis* showed zone of inhibition of 23±1 mm, while *Staphylococcus epidermidis* strain, exhibited inhibition value of 24±3 mm. Earthworm extract didnot express any inhibition effect on remaining four strain of bacteria viz. *Streptococcus pyrogenes, Enterococcus faecalis, Enterobacter aerogenes* and *Escherichia coli*. Therefore it can be concluded that earthworm extract has antibacterial activity against some bacteria, while others may not be affected.
Similar to the antibacterial activity, anti-fungal activity was also evaluated by measuring zone of inhibition on SDA plates by well-diffusion method. No antifungal activity was observed against two fungi tested in the present study, *Candida albicans* and *Aspergillus niger*.

**6.7 Wound healing activity:**
The wound healing activity of earthworm extract was studied by topical application of ointment of test material on excised wounds of experimental rats. In addition to recording observations on wound contraction percentage and epithelialisation time, wounded tissue was also subjected to histological investigations and observations.

The rats treated with Betadine (positive control as standard healing drug) showed satisfactory healing influence with 97.95±0.30% wound contraction percentage and epithelialisation time of 16.80±0.21 days. The wounded rats treated with EW extract ointments showed progressive healing process and even better results than Betadine were obtained. Rats treated with 50 mg earthworm extract ointment showed 100% wound contraction percentage and epithelialisation time of 14.17±0.30 days. Results of lower (25 mg) and higher (100 mg) doses of earthworm extracts showed comparatively lower influence on these parameters.

The results of histological study were consistent with parameters (wound contraction percentage and epithelialisation time) of wound healing. From careful observations of histologically prepared slides, it was confirmed that best results of healing were depicted with 50 mg of earthworm extract ointment; showing the presence of squamous epithelium, fibro-collagenous tissues, containing significantly higher amount of collagen fibers, lesser lymphocytes, copious tiny blood vessels, and enormous and well formed sweat glands and hair follicles, all of these parameter are considered as indication of complete healing. In other groups of rats varying degrees of healing conditions were noticed. The healed skin of control rats which have revealed least wound contraction percentage and longer epithelialisation time on day 16<sup>th</sup> depicted immature squamous epithelium layer, immature fibroblast, oedema, hair follicles and lymphatic infiltrates or inflammatory infiltrates (leucocytes) and absence of sweat glands.
The healed skin of negative control rats showed better results of wound contraction percentage and longer epithelialisation time also revealed better healing histological parameters *i.e.*, almost mature epidermis and fibro-collagenous tissues, lymphatic infiltrates, but sweat glands and hair follicles were still not observed. In Betadine treated rats presence of immature epidermis, immature fibroblast, mild lymphatic infiltrates, less collagen fibres and connective tissues were noticed. But better conditions of development of sweat glands and hair follicles were observed.

In EW extract ointment treated different groups, results of histology revealed that in 25 mg EW extract ointment group immature squamous epithelium, immature fibro-collagenous tissues, less lymphatic infiltrates were shown whereas hair follicles and sweat glands were enormous and well formed. In 100 mg EW extract based ointment group immature epidermis, mild lymphatic infiltrates, fibrous-connective tissues which contain less collagen fibres, were observed. Hair follicle and sweat glands were well formed.

6.8 Fibrinolytic, Proteolytic and Fibrinogenolytic activity:

The fibrinolytic activity was evaluated on plasminogen free and plasminogen rich fibrin plates by well-diffusion method. Observations on measurement of zones of lysis revealed that the fibrinolytic activity of EW extract was significantly higher (1.27 times / 21.49%) on plasminogen rich plate than on plasminogen free fibrin plate. This might be due to activation of plasminogen to plasmin by some factors of the EW extract. It was further observed that the fibrinolytic activity of EW extract was much higher than that of streptokinase (standard fibrinolytic drug).

The fibrinolytic activity was also demonstrated by euglobulin clot lysis time (ECLT) assay. The blood from healthy human volunteers was subjected to euglobulin precipitate followed by incubation with sample. It was found that ECLT values of EW extract were much lower than those of streptokinase indicating a faster lysis of euglobulin due to higher fibrinolytic activity.
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The proteolytic activity of earthworm extract using casein as substrate was observed to be 1.274±0.04 Units/ml and the specific enzyme activity was 18.20±0.5 Units/mg of sample protein.

The fibrinogenolytic activity of the earthworm extract was also demonstrated using fibrinogen as substrate and analyzing the end products by SDS-PAGE. Complete degradation of Aα and Bβ-chains of fibrinogen was observed in a very short time (1 min) and the γ-chain was completely hydrolyzed in 1 hour. No significant protein bands could be observed after 180 minutes indicating an excellent fibrinogenolytic activity.

6.9 Conclusion:

An earthworm (Lumbricus rubellus) based drug has already been launched at international scenario that is useful in prevention of cardiovascular complications. It has also been reported that similar therapeutic properties are present in Eisenia fetida. The present study on "Evaluation of pharmaceutical significance of the earthworm Eudrilus eugeniae" was conducted because this may lead to new drug discovery from a renewable natural resource. Following can be drawn from the findings of the study:

1. The most potential biological property of bio-therapeutic agents is their antioxidant and free radical scavenging activity which helps in getting rid of debris and toxins generated by natural cell death, infections, pollution etc. and to promote natural functioning of all body systems. In vitro experiments (DPPH radical assay, reducing power assay and total phenolic content assay) revealed that earthworm (Eudrilus eugeniae) extract possesses enough activity of this kind.

2. Two in vivo experiments (carageenan induced paw oedema and cotton pellet induced granuloma pouch) were conducted on experimental rats to demonstrate anti-inflammatory activity of EW extracts. Both of these treatments were found to cause acute and chronic inflammatory reactions. The inflammation was found to be significantly suppressed by pre-treatment with standard anti-inflammatory drug indomethecin and EW extracts.
3. Above anti-inflammatory experiments were additionally used for biochemical demonstration of parameters of oxidative stress markers like reduced glutathione (GSH), thiobarbituric acid reactive substances (TBARS), super oxide dismutase (SOD) and catalase activities. The results have demonstrated that the levels of oxidative stress markers were significantly higher in control than experimental (Indomethecin and EW extract treated) animals indicating significant oxidative stress removing activity.

4. The level of cytokines TNF-α and IL-10 were used to demonstrate as marker of inflammatory and anti-inflammatory conditions in both acute and chronic model of inflammation. Declining levels of TNF-α and increasing trend of IL-10 indicate anti-inflammatory effect of EW extract and indomethacin.

5. Presence of anti-pyretic activity in EW extract was also proved in Yeast induced pyrexia when different concentration of EW extract lower the body temperature in experimental rats.

6. Testing of antibacterial activity has demonstrated that of earthworm extract can inhibit the growth and multiplication of some strains (both Gram positive and Gram negative) of bacteria, while the extract was not effective against some other strains of bacteria. Hence EW extract has a good potential to develop as a new antibacterial drug.

7. **In vivo** experiments rats have revealed that earthworm based ointment helps in faster healing of excised wounds. The treated animals showed high wound contracting percentage, reduced epithelisation time as compared to control rats.

8. Fibrinolytic activity of EW extract was proved by fibrin plates (plasminogen free and plasminogen rich) and euglobulin clot lysis time assay (ECLT). Fibrinolytic activity on fibrin plates was much higher than standard fibrinolytic drug streptokinase. Euglobulin clots were lysed much faster with EW extract than comparison to streptokinase, which indicate higher fibrinolytic activity.

9. In proteolytic activity, it was demonstrated that EW extract has potential to degrade other protein substrate like casein in addition to fibrinogen/fibrin.
10. Degradation of fibrinogen by EW extract has indicated an excellent fibrinogenolytic activity.

11. These conclusions are in line with earlier finding on *Lumbricus rubellus* and *Eisenia fetida*.

12. The outcome of the study may help, in some way, in development of new drugs based on renewable bio-resources which may free from the side effects and may be more effective for prevention and treatment of certain diseases.

13. Further, more, in depth, studies are required particularly dealing with anti-coagulant and clot dissolving properties of EW extracts and to improve the technique of sample preparation.