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Ethnozoological importance of *Eisenia fetida* and experimental validation of its anticancer activity in ascites Dalton's lymphoma (DL) bearing mice

Manash Pratim Borah[1*] and Surya Bali Prasad[2]

Abstract

Eisenia fetida, commonly known as the red earthworm, holds significant ethnozoological importance due to its traditional use in various cultures as a medicinal agent. This study aims to explore the potential anticancer effects of *Eisenia fetida* paste (EFP) and evaluate its therapeutic efficacy in mice with ascites Dalton's lymphoma (DL). The EFP extract demonstrated a significant antitumour effect, possibly by initiating programmed cell death and mitochondrial changes in the tumour cells. The viability of tumour cells exhibited a reduction over time due to EFP treatment. Comparative analysis with the reference drug cisplatin revealed that EFP exhibited fewer or no adverse effects on mutagenicity, hepatotoxicity, and nephrotoxicity in tumour-bearing hosts. EFP treatment was found to show progressive increase in the quantity of apoptotic DL cells over time suggesting a time-dependent impact on the induction of apoptosis in the treated groups. These results imply that EFP might serve as a safer substitute for cancer treatment and hold promise for developing new and improved therapeutic strategies against cancer. The main implication of the study is that EFP might serve as a safer substitute for cancer treatment. It suggests a promising avenue for developing new and improved therapeutic strategies against cancer, with the potential to enhance treatment outcomes while minimising adverse effects.

Keywords Ethnozoology, *Eisenia fetida*, Anticancer, Antitumour

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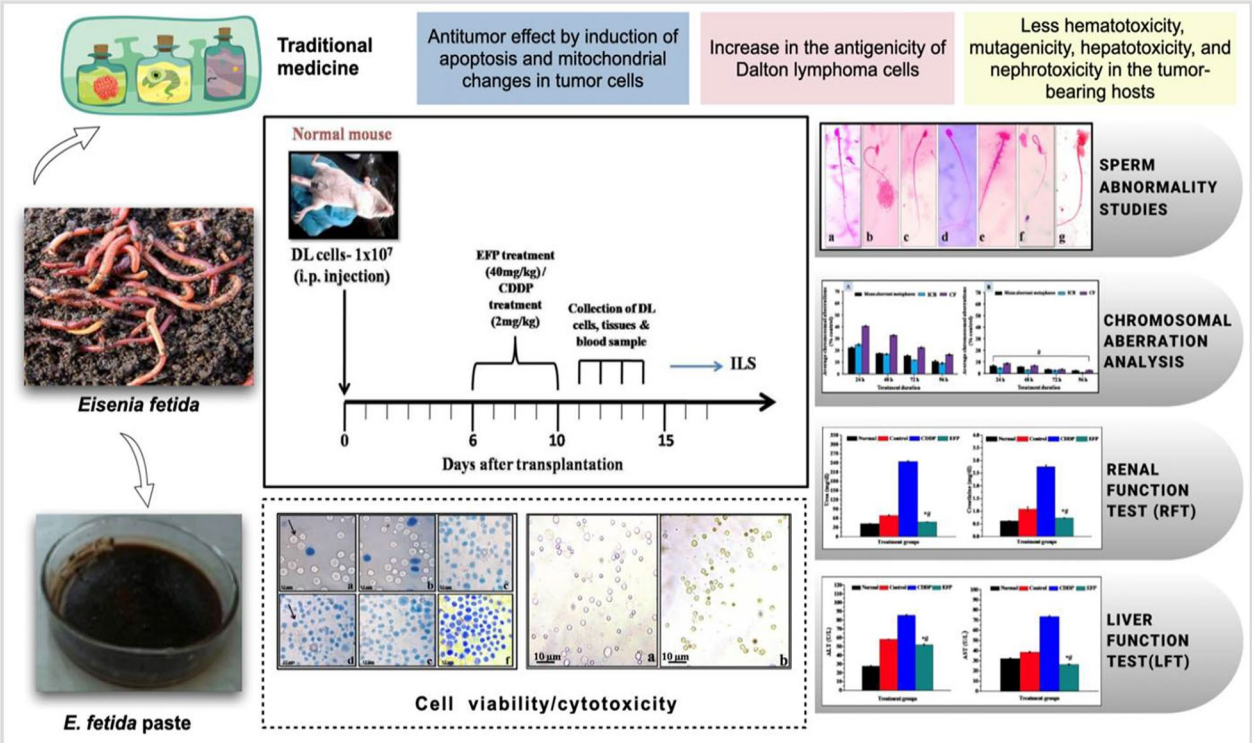
Graphical abstract



Background

Approximately 19.3 million fresh cancer cases across the globe were estimated by the Global Cancer Observatory (GLOBOCAN) in 2020, with China ranked first, followed by the USA and India in third. GLOBOCAN's predictions indicated that the figure of cancer cases in India is predicted to rise significantly by 57.5% from the number of cases reported in 2020 [31]. Cancer can arise from the abnormal growth and division of cells in various tissues throughout the body, giving rise to over a hundred distinct types of cancer. These different types of cancer can exhibit significant variations in their behaviour and how they respond to treatment [13].

The indications of cancer differ based on the disease's type and stage. Common signs may comprise persistent unexplained weight loss, pain, fatigue, changes in the skin, lumps or thickening of tissues, persistent cough or hoarseness, alteration in bowel or bladder patterns, and difficulty swallowing, among others [10, 39, 44]. The diagnosis of cancer involves different methods, such as physical examinations, medical history reviews, diagnostic imaging procedures (such as CT scans, X-rays, MRIs, or PET scans), laboratory tests (like blood tests or biopsies), and genetic testing [18, 21, 24, 36, 52]. The proper treatment options mainly depend on factors such as the cancer's stage, type, and location, alongside the patient's overall well-being [32, 37]. Among the treatment modalities radiation therapy, surgery, chemotherapy, hormone therapy, immunotherapy, and targeted therapy are the most common [7, 49]. Often, a combination of treatments is used to achieve the best outcome.

Across history, natural compounds have held a substantial role in uncovering anticancer medications. Several commonly used cancer treatments have their origins in nature-derived sources like irinotecan, vincristine, paclitaxel, and etoposide derived from plants, while mitomycin C and actinomycin D come from bacteria and marine bleomycin [14, 15, 26]. These compounds are crucial in cancer therapy and are expected to retain their importance in the coming years. Extensive research has been conducted on phytochemicals, revealing their anticarcinogenic properties through their influence on multiple mechanisms resulting in initiation, development, and succession of cancer [27, 51]. However, compared to plants, research on animal-derived products for cancer treatment is relatively less. The traditional medicinal

practices in Algeria suggested consuming diverse wild animal species, including *Atelerix algirus*, *Varanus griseus*, *Corallium rubrum*, and *Cancer pagurus,* as remedies for various types of cancer [54]. Many communities in North-Eastern parts of India were also found to use different body parts of essential animal species such as gall bladder, horn, fur, intestine, bone, liver, blood, fat, heart as well as tongue as anticancer agents. The Wangsho and Tangsha tribes of Arunachal Pradesh, the Koch-Rajbonshi, Kalita, Ahom, Chutia, Tea tribes of Assam, and Rongmei of Manipur used various types of mammals, birds, reptiles, fish, molluscs, arthropods as home remedies for cancer [8, 29, 58]. While numerous researchers have recorded the therapeutic benefits of various plant species from Assam, there needs to be more comprehensive documentation regarding the traditional utilisation of animals in treating ailments specific to the region. The significance of blister beetles (*Mylabris cichorii*) in zootherapeutics among the Karbis residing in the Karbi Anglong district of Assam has been documented, along with its strong potential in combating ascites Dalton's lymphoma and Ehrlich ascites carcinoma, showing powerful anticancer effects [45, 46, 57, 58]. It was found that bee venom (melittin), scorpion toxin (Chlorotoxin), has significant inhibitory effects on the EGF-induced invasion, proliferation, migration of breast cancer cells [38]. In another study, it was found that marinobufagin exhibits its anticancer effects against colorectal carcinoma both in vitro and in vivo [20]. In this study, the use of a particular earthworm (*Eisenia fetida*) was assessed for its anticancer potential in the laboratory against the experimental malignant tumour- murine ascites Dalton's lymphoma. An initial ethnozoological survey of the adjacent areas of the few wildlife sanctuaries/national park of Assam and the information of some animals used against cancer-suspected disease, an earthworm species were selected to evaluate its anticancer potentials against a murine malignant tumour. The aim of this study is to contribute to the existing ethnozoological knowledge and explore the potential of EFP as an alternative and potentially safer anticancer treatment for future research and therapeutic developments in cancer treatment.

Results

Screening of antitumour activity of earthworm pastes/extracts

Different doses of *Eisenia fetida* paste (EFP) were used to check their antitumour activity regarding alterations in the viability of mice bearing ascites Dalton's lymphoma under different experimental groups. From the different doses of EFP (10, 20, 40, 80, and 160 mg/kg body weight/day) used, the highest increase in the life span in mice carrying ascites Dalton's lymphoma (in mice with DL was noted to be 49.47% with a dose of 40 mg/kg body weight (Table 1).

Thus, from the preliminary screening of antitumour activity at different doses of EFP, it was derived that the EFP dose of 40 mg/kg body weight has enhanced curative efficiency against murine ascites Dalton's lymphoma. The patterns of comparative changes in ILS after EFP and cisplatin treatments are shown in Fig. 1.

Cell viability/cytotoxicity study

The result of EFP on the DL cell viability and spleen cells was checked using the trypan blue exclusion test. EFP treatment caused a time-dependent reduction in the case of tumour cell viability (Figs. 2 and 3). There was less cytotoxicity in spleen than in tumour cells (Figs. 4 and 5). These observations indicate that normal cells exhibited a lower sensitivity to the EFP extract when compared to tumour cells.

Table 1 Antitumour activity of EFP against murine ascites Dalton's lymphoma

Earthworm extracts	Dose (mg/kg body weight/day)	Life span (days) (mean ± SD)	ILS %	Survival on day 30 Survival/total
EFP	Control	19.0 ± 0.816	–	0/10
	10	20.2 ± 1.249	6.32	0/10
	20	23.4 ± 2.498	23.16*	1/10
	40	28.4 ± 1.955	49.47*	3/10
	80	24.7 ± 1.417	30.00*	1/10
	160	17.2 ± 1.619	− 9.47	0/10

Asterisk indicates a significant level of ILS%, which can be considered to have an enhanced curative effect against Ascites-Dalton's lymphoma

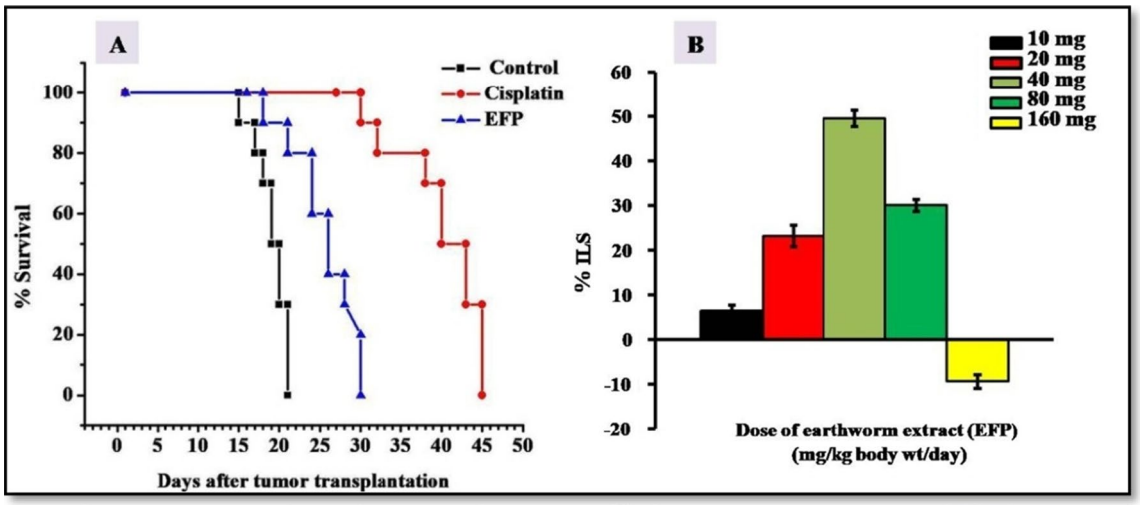


Fig. 1 **A** Survival pattern and **B** percent increase in life span (%ILS) of tumour-bearing mice after EFP treatment. Results are expressed as mean SD ($n = 5$). The control refers to untreated tumour-bearing mice, while CDDP stands for cis-diamminedichloroplatinum (II) or cisplatin

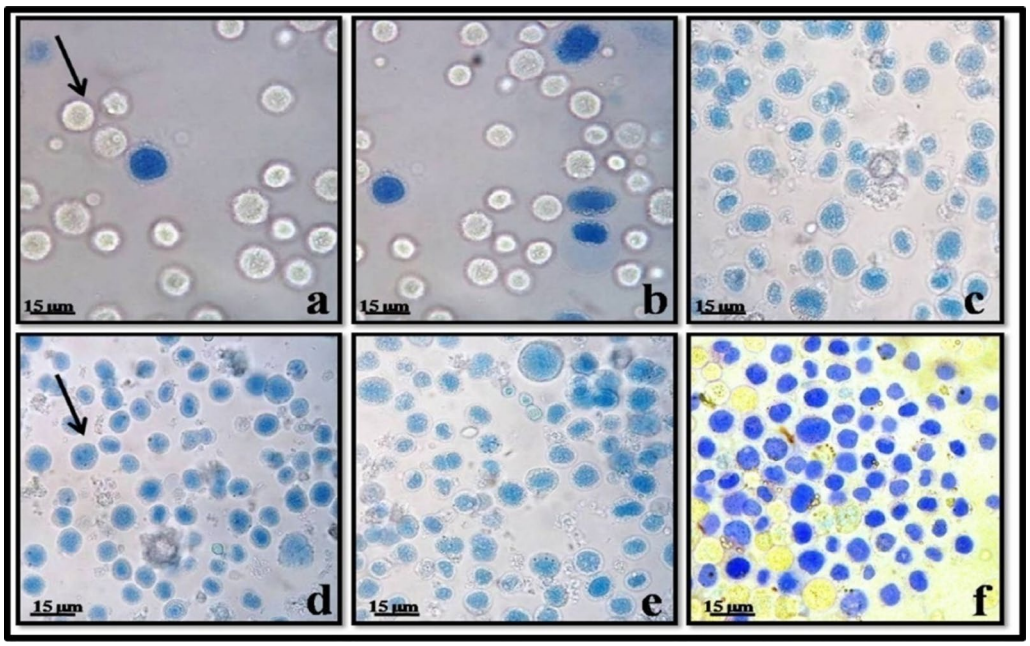


Fig. 2 Trypan blue exclusion test to find the viability of DL cells with different treatment conditions. **a** DL cells treated with drug vehicle as a control; **b**–**e** EFP treatment for 24–96 h; **f** CDDP treatment at 96 h. Colourless viable cells contrast with blue-stained deceased cells. The black arrow points out the viable cells in **a** and blue-stained deceased cells in **d**

Apoptosis study

(AO/EtBr) staining

The DL cell nuclei as control were round-shaped with consistent green fluorescence, indicating viable cells (Fig. 6a). On the other hand, in all the treatment groups, different apoptotic characteristics were noted in DL cells at varying time points (Fig. 6b-e). Various apoptotic features were found in the DL cells of CDDP-treated mice also (Fig. 6f). In each of the treated groups (CDDP and EFP treatment), the quantity of apoptotic DL cells showed a time-dependent increase (Fig. 7).

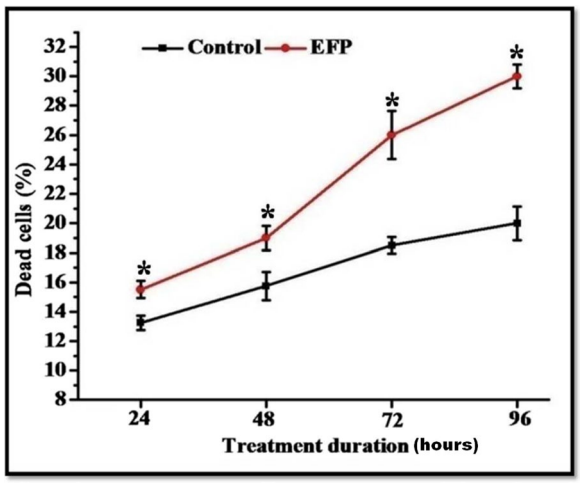


Fig. 3 Quantitative changes in the dead DL cells from mice over diverse treatment conditions determined by the Trypan blue exclusion analysis. Results are expressed as mean SD ($n=4$)

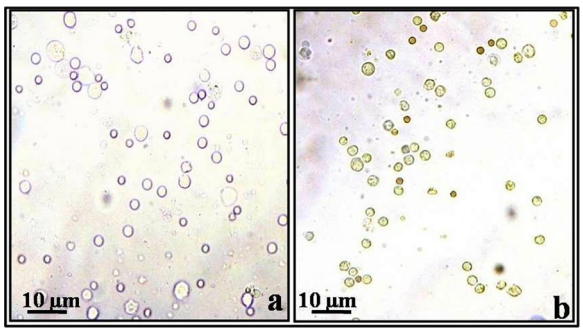


Fig. 4 Trypan blue exclusion analysis to check the viability of spleen cells. **a** Control cells are treated with only the drug vehicle; **b** 96 h of EFP treatment. Viable cells are colourless, and non-viable cells stain blue

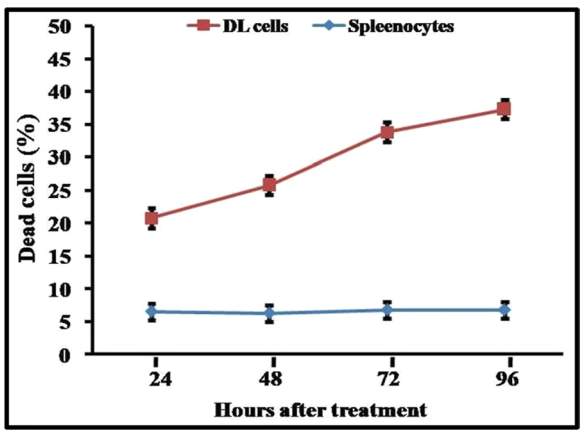


Fig. 5 Changes in the visibility of mice's DL and spleen cells (splenocytes) after EFP treatment. The results are expressed as mean SD ($n=4$)

Scanning electron microscopy and Transmission electron microscopy of DL cells

Scanning electron microscopy (SEM)

DL cells used as control were almost round shape with few projections in the membrane and evenly scattered ruffles over the surface (Fig. 8). CDDP-treated tumour-bearing mice showed a decline in ruffles or microvilli on DL cells and at 96 h of treatment membrane fusion and plasma membrane deformities were observed (Fig. 8f). The development of deformities in DL cells including loss of microvilli from the surface in the initial hour of treatment, the appearance of membrane bleb, as well as cell shrinkage were also observed in EFP-treated tumour-bearing mice (Fig. 8b–e).

Transmission electron microscopy (TEM)

TEM study was done in DL cells to analyse the possible alteration and changes inside the cells subjected to varying treatment conditions (Fig. 9). DL cells used as control showed cellular processes uniformly distributed over the cell surface along with large clear, uniform nuclei, uniform chromatin, smooth plasma damage and disruption of mitochondrial membrane along with cell membrane and distinct normal mitochondrial features with regular cristae (Fig. 9a, b). CDDP treatment in mice caused mitochondrial damage, cytoplasmic vacuolation, the disappearance of membrane processes and nuclear fragmentation in DL cells (Fig. 9c, d).

Assessment of toxicity of earthworm pastes/extracts [EFP]

Mutagenic studies

Sperm abnormalities Different morphological abnormalities were observed in sperms of mice after CDDP and EFP treatment. The categories and forms of sperm anomalies triggered by CDDP encompass head without a hook, looping midpiece, head like a balloon, diffused head, amorphous head and so on. EFP-treated tumour mice also show sperm abnormalities like banana-shaped heads, coiled necks, hammer-shaped heads, and bulged heads in the tumour-bearing mice (Fig. 10b-g). However, mice with tumours treated with EFP showed reduced sperm abnormalities in the tumour-bearing hosts compared to CDDP treatment (Table 2; Fig. 11).

The outcomes are shown as mean ± SD. The analysis was performed with one-way ANOVA long with Tukey's test, with a sample size of 4. A $p \leq 0.05$ level of significance was employed in comparison with CDDP. A normal mouse lacks a tumour and any treatment; CDDP = Cisplatin. BSH = banana-shaped head, BLH = balloon-like head, HSH = hammer-shaped head, CN = coiled neck and BH = bulged head. The examination of sperm was conducted on the fifth day of the treatment.

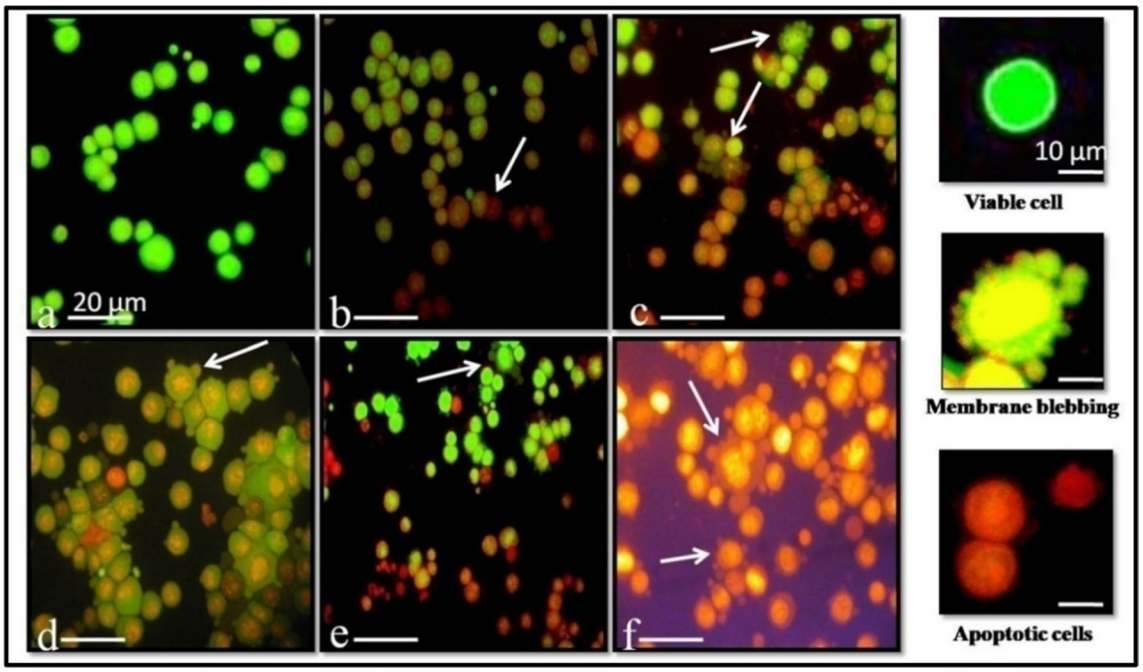


Fig. 6 Fluorescence-based apoptosis analysis using AO/EB staining in DL cells. Control DL cells **a**; EFP-treated for 24, 48, 72 and 96 h **b**, **c**, **d** and **e**; and cisplatin for 96 h **f**. Regular arrows showing apoptotic features with membrane blebbing/folding

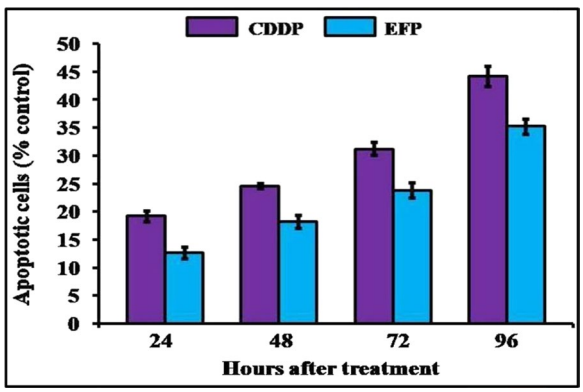


Fig. 7 Pattern of percentage apoptotic DL cells (apoptotic index) after treatment with CDDP and EFP. Results are expressed as mean SD ($n = 5$)

Chromosomal aberration analysis A few aberrations, such as isochromatic break and chromosomal fragments, were detected within the bone marrow cells following EFP treatment (Fig. 12). The highest occurrence of the aberration mentioned above was observed after treatment of 24 h. There was a considerable decrease in the mean aberrant metaphase under 24–96 h treatment in both CDDP and EFP-treated groups. CDDP treatment in the tumour-bearing mice showed more chromosomal aberrations than the EFP-treated tumour-bearing mice (Fig. 13).

Renal function (RFT) and Liver function (LFT) tests

Renal function test (RFT)

Serum creatinine, as well as urea level, were determined to examine the changes in renal toxicity. The serum creatinine and urea level measurement in the tumour-bearing control showed a slight variation from their normal counterpart. EFP-treated tumour-bearing mice showed a reduction in serum creatinine and urea levels compared to the control tumour group and were almost equalised with normal mice. However, administration of CDDP resulted in a notable rise in serum creatinine and urea levels compared to the control group (Table 3; Fig. 14.)

Liver function test (LFT)

The alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity were examined to measure the toxicological changes in hepatic functions in hosts exposed to different treatment conditions.

The control mice showed increased activity of both AST and ALT as compared to normal (Table 4). However, compared to the control, EFP treatment exhibited a minimal alteration in ALT and AST activity. On the other hand, CDDP treatment displayed a considerable increase in ALT and AST activity compared to control mice (Table 4; Fig. 15).

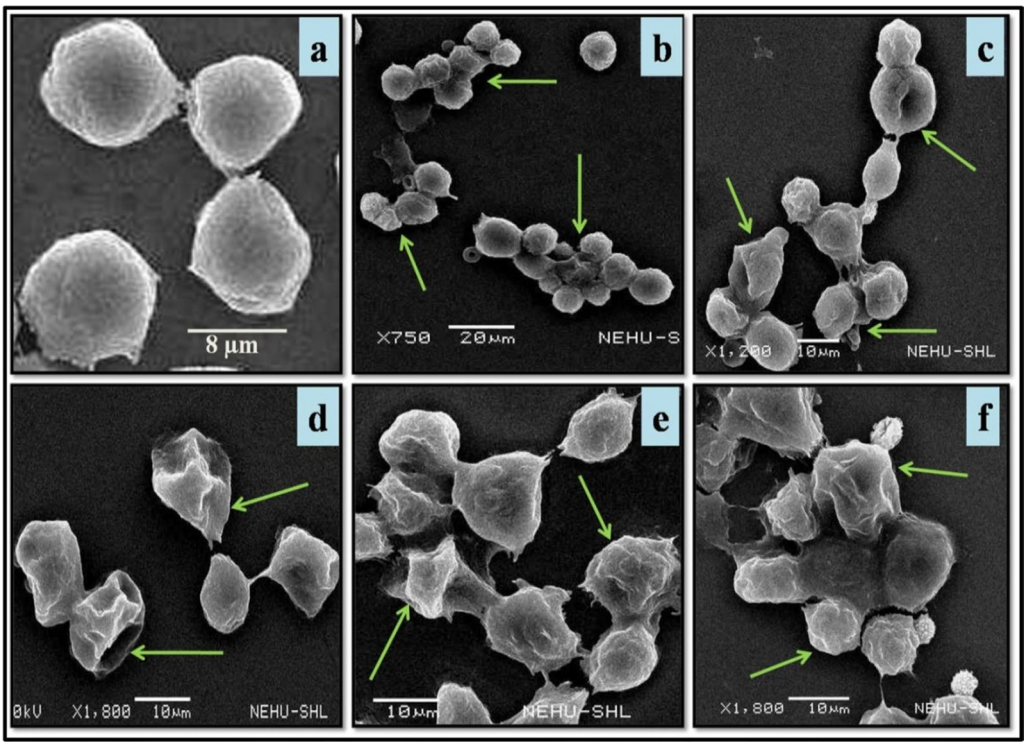


Fig. 8 SEM images including Dalton's lymphoma (DL) cells and control cells **a**, *Eisenia fetida* paste (EFP)-treated cell at 24–96 h **b–e**, Cisplatin (CDDP) treatment at 96 h **f**. The control DL cells exhibited a round shape with projections in the membrane and evenly distributed regular ruffles over the surface. Important cellular appearances on the cells, like membrane blebbing, cell membrane fusion, and cell deformities, are indicated by green arrows

Discussion

Ascites Dalton's lymphoma, also known as T cell lymphoma, has been consistently utilised as a significant model for experimental murine malignant tumours [40, 56]. The reliable measure to assess a drug's antitumour efficacy is considering the extension of the lifespan of the treated hosts with tumours (Prasad, Nicol et al. 2010, [56]. Examination of the survival capability of splenocytes and DL cells in various in vivo treatment scenarios indicated that EFP treatment elicited more significant cytotoxicity in DL cells compared to splenocytes. This suggests that EFP affects normal cells with lower sensitivity than its impact on DL cells (Figs. 4 and 5). The anticancer potential of *Eisenia fetida* extract/paste or compound isolated from it has been observed on a variety of other cancer cell lines such as HCT116 human colorectal cancer, MCF-7 breast carcinoma [34, 48], HeLa and LTEP-A2 cells [25]. This is the first study to look at the antitumor activity of an earthworm extract (Eisenia fetida paste, EFP) from Northeast India.

Apoptosis is an ordered and orchestrated cellular process controlled by a genetically regulated mechanism in different physiological and pathological conditions. It is a strongly held multi-step pathway responsible for cell death to control the cell number [28, 59]. It has a vital role in the development of tumour-related diseases along with other different illnesses. Unrestrained growth and malfunction of apoptosis mechanisms form essential components in the growth and advancement of a malignant tumour [9, 43]. Apoptosis in the cells is identified by the formation of membrane blebs, cell and organelle contraction, DNA fragmentation, nuclear condensations, and cell disintegration at the end [17]. Numerous cancer chemotherapeutic medications have been documented to trigger apoptosis in cancerous cells [22]. Assessments based on the TEM-, SEM- and AO/EtBr-based fluorescence microscopy are excellent indicators for confirmation of apoptotic features [42]. The present AO/EtBr-based fluorescence study of DL cells revealed that the EFP treatment caused severe membrane blebbing, cell shrinkage, cell membrane disintegration, and other apoptotic features in the DL cells in a time-dependent fashion. This may concur with an earlier report from the study using earthworm extract/paste of *Lampito Mauritius* showing similar kinds of apoptotic features in HT29 colon cancer cells [35]. As

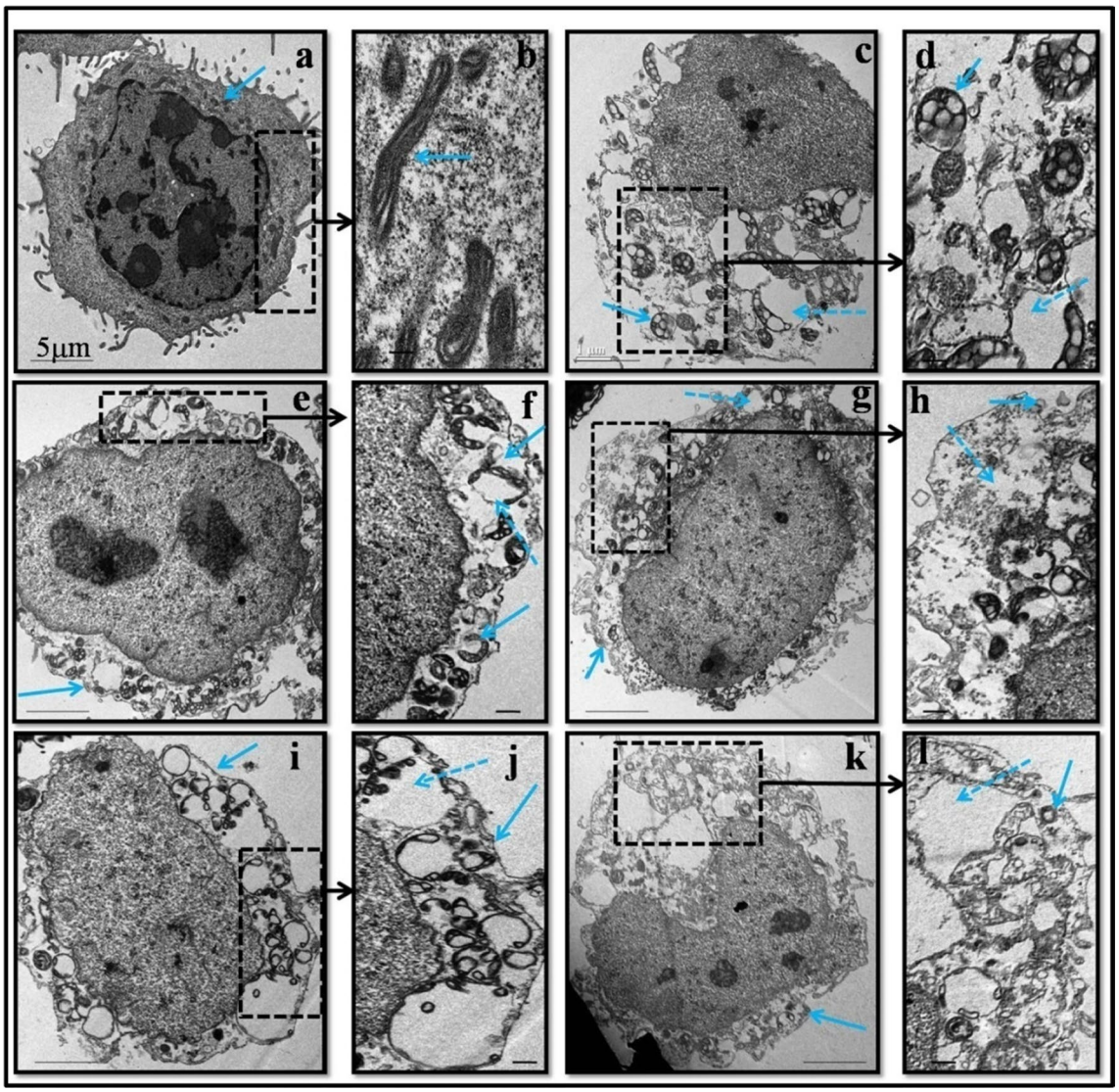


Fig. 9 Ultrastructural features of DL cells. The blue arrow in the control shows DL cells **a** with distinct mitochondria cristae, The blue arrow showing mitochondrial damage and nuclear fragmentation after CDDP treatment from 96 h **b**, **c** and **d**, EFP treatment for 24-96 h **e–l** showed pronounced vacuoles and mitochondrial EFP-treated tumour-bearing mice also developed similar structural features in DL cells as observed for CDDP treatment as indicated by the blue arrows. The mitochondria showed rounded deformed cristae and a few vacuoles at 24 h of treatment (Fig. 9**e**–**f**). At 48 h to 96 h of EFP treatment, the DL cells showed fragmented nuclei, prominent vacuoles, membrane disorganisation, and disordered organisation of mitochondrial cristae with severe damage observed as indicated by the blue arrows (Fig. 9 **g–l**)

observed in EFP-treated groups, the reference drug cisplatin treatment also led to the development of apoptotic features in DL cells.

Surface morphological and ultrastructural changes are significant indicators of cellular damage, measured as distinct indicators of apoptosis. SEM studies showed a sequence of alterations on the surface of the DL cells following treatment with EFP and cisplatin. Cisplatin treatment for 96 h showed membrane blebbing, cell shrinkage, membrane projection (microvilli) loss and developed cell deformities compared to control DL cells, which are circular with membrane extensions and undulations in the cell surface. EFP treatments also caused similar surface morphological changes in DL cells. The above alterations in the cell surface morphology after treatment with EFP may indicate the appearance of apoptotic characteristics in DL cells.

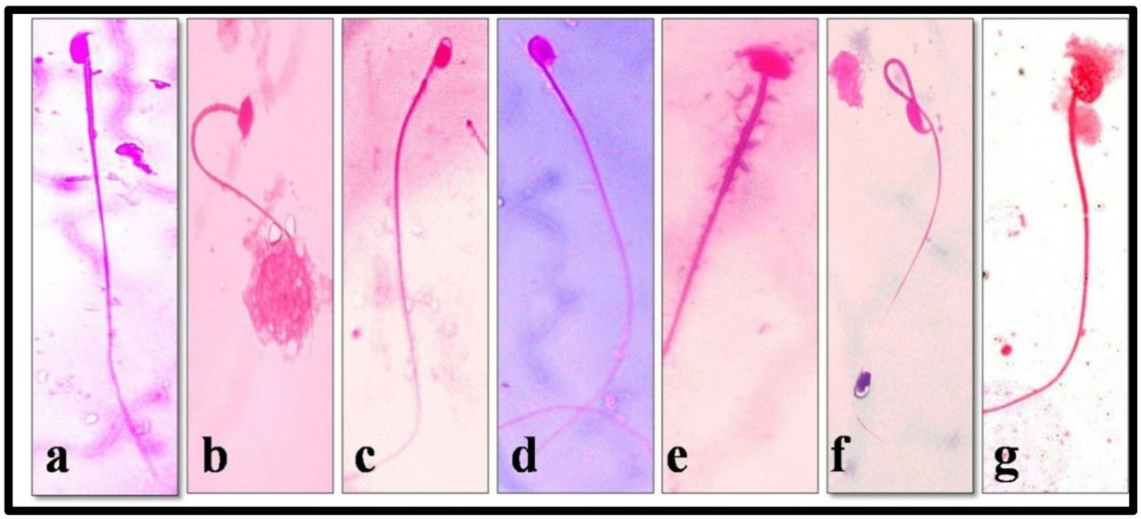


Fig. 10 Illustrative images depicting diverse forms of morphological abnormalities in mouse sperm under varying treatment conditions, **a** normal, **b** banana-shaped head, **c**, **d** balloon-like head, **e** hammer-shaped head, **f** coiled neck **g** bulged head

Table 2 Numeric assessment of diverse categories of morphological abnormalities in sperm observed across varying treatment conditions

Treatment	No. of sperms observed	No. of abnormal sperms	BSH	BLH	HSH	CN	BH	Mean of abnormal sperms
Control	500	4	1	0	0	2	1	0.8 ± 0.1
CDDP-treated	500	33	11	6	4	7	5	6.6 ± 0.2
EFP-treated	500	14	3	2	2	4	3	2.8 ± 0.34*

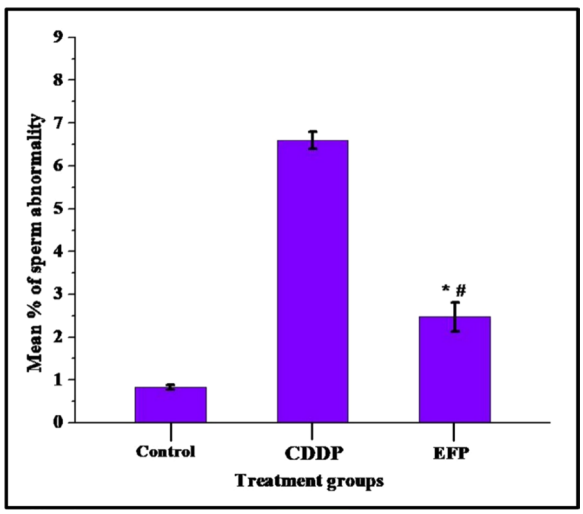


Fig. 11 Changes in the prevalence of sperm abnormalities resulting from the administration of CDDP (Cisplatin) and EFP (Eisenia fetida paste) treatment. The findings are expressed as mean SD ($n = 4$). *$P \leq 0.05$ as compared to the chosen control; #$P \leq 0.05$ when compared to CDDP

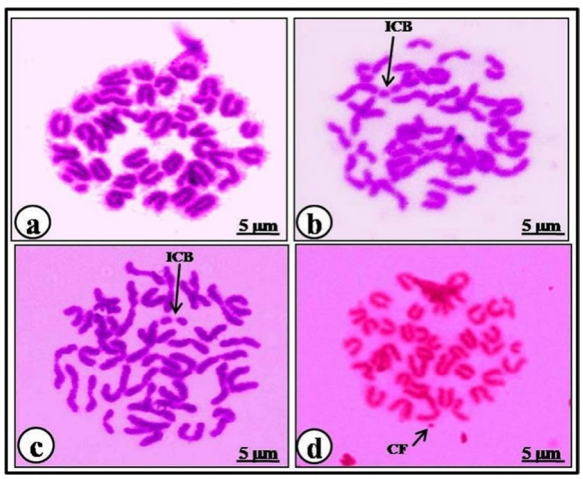


Fig. 12 Illustrative images depicting bone marrow metaphase chromosome spreads. **a** Typical array of chromosomes, along with various categories of chromosomal abnormalities, including **b** & **c** Isochromatid break (ICB) and **d** Chromosomal fragment (CF)

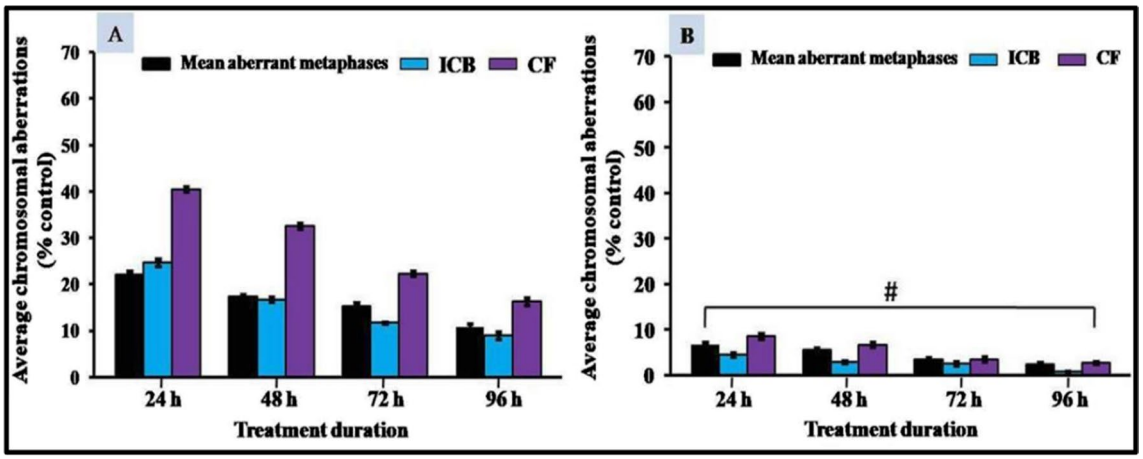


Fig. 13 Statistical examination of mean deviant metaphases, isochromatic breaks (ICB) and chromosomal fragment (CF) after treatment with CDDP **A** and EFP **B**. Results are expressed as mean SD ($n = 3$). The symbol "#" indicates significance at $p \leq 0.05$ compared to CDDP

Table 3 Levels of serum creatinine and urea across various experimental conditions

Treatment groups	Creatinine (mg/dL)	Urea (mg/dL)
Normal	0.62 ± 0.01	42.76 ± 0.56
Control	1.09 ± 0.08	69.56 ± 1.91
CDDP-treated	2.75 ± 0.06	244.67 ± 2.51
EFP-treated	0.74 ± 0.01*#	48.68 ± 0.29*#

Mean ± S.D. values are expressed with ANOVA, $n = 3$, $^*P \leq 0.05$ comparing with the consequent control; $\#P \leq 0.05$ vs. CDDP. Blood samples were taken on the 5th day post CDDP or EFP treatment. The normal mice is devoid of tumour as well as any treatment. The control is the mice with tumour and without treatment, EFP = *Eisenia fetida* paste; CDDP = *cis*-diamminedichloroplatinum (II) or cisplatin

TEM studies revealed damage in the cell membrane structure, cytoplasmic vacuolation, mitochondrial damage, etc., as prominent features observed in DL cells. Mitochondria is crucial in controlling apoptosis, maintaining malignant phenotype, and mutagenesis [1, 16, 33] . In the present study, the ultrastructural examination of control DL cells showed distinct mitochondrial features with regular cristae and uniform nuclei, smooth plasma membrane and uniformly distributed cellular processes over the cell processes. Structural abnormalities like mitochondrial membrane disruption reduced and damaged cristae cell cytoplasmic vacuolation, the disappearance of membrane processes and fragmentation of the nucleus were detected in DL cells at 96 h of cisplatin treatment in vivo. Treatment with EFP also showed severe structural deformities in mitochondria and other cellular organelles. Disruptions in mitochondrial structure and function in developing tumours have been associated with the participation of hypoxia-inducible factor (HIF-1α) in activating the glycolytic enzymes

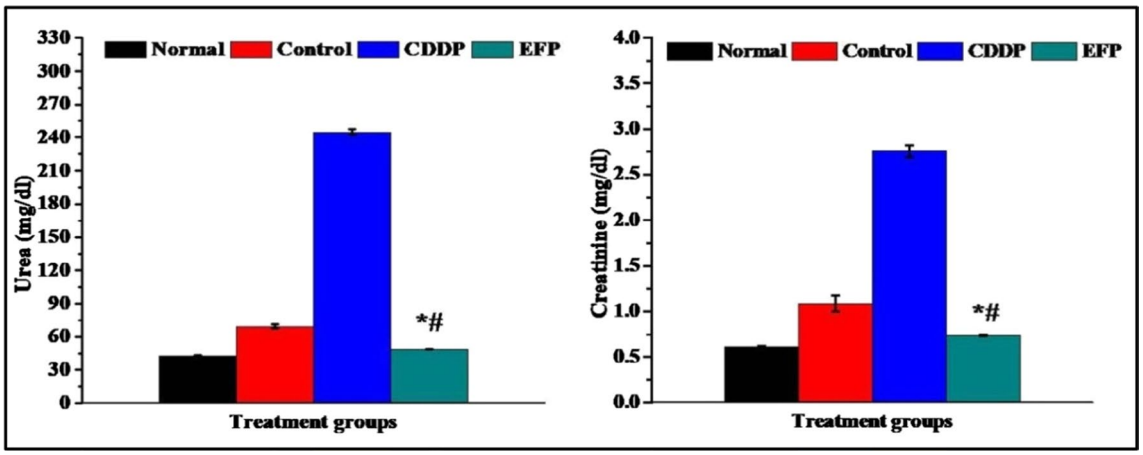


Fig. 14 Changes in serum urea and creatinine levels (mg/dL) of mice with tumours under various treatments. Mean ± S.D. ANOVA was used to express the results as mean SD ($n = 3$), $^*P \leq 0.05$ when evaluated with the corresponding control; # $P \leq 0.05$ when compared with CDDP

Table 4 Levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity across various experimental scenarios

Treatment groups	ALT (SGPT) U/L	AST (SGOT) U/L
Normal	27.62 ± 0.52	32.10 ± 0.54
Control	58.17 ± 0.21	38.50 ± 0.65
CDDP-treated	85.71 ± 0.78	73.71 ± 0.51
EFP-treated	52.10 ± 0.83*#	26.44 ± 0.42*#

Mean ± S.D. values are presented with ANOVA, $n = 3$, *$P \leq 0.05$ as compared to the chosen control; #$P \leq 0.05$ when compared to CDDP. Blood was collected on the 5th day after CDDP or EFP treatment. The normal mice is devoid of any tumour or treatment condition and the control mice is with the tumour without treatment, EFP = *Eisenia fetida* paste; CDDP = *cis*-diamminedichloroplatinum (II)

under various oncogenic stimulations [30]. Moreover, the damage in the mitochondria may also be because of the malfunction of the respiratory chain and the decline in membrane potential, resulting in the disturbance of cellular energy; AMPK plays a pivotal role in upholding energy balance during heightened stress conditions. Inhibition of the AMPK pathway occurs in this context [23]

In the present study, different chromosomal abnormalities, including isochromatic breaks, chromatid breaks, exchanges, unions of sister chromatids, and chromosomal fragments, were identified in bone marrow following cisplatin treatment, as documented in previous studies. However, only a few isochromatic breaks and chromosomal fragments were found in the EFP treatment compared to the cisplatin treatment. In the treatment conditions, the total frequency of aberration in metaphase and CA was highest after 24 h of treatment, gradually decreasing as the treatment progressed to later time points. The time-dependent decrease in CA after the treatment could be because of the death of damaged cells or post-replication repair processes in the cells [12]. The quantitative analysis of mean aberrant CA in EFP treatment showed a minimal chromosomal aberration in bone marrow cells compared to cisplatin treatment, suggesting that EFP is much less mutagenic. These findings may also be supported by some naturally occurring cytotoxic compounds such as propolis and bee venom [2, 3]. Thus, the assay results of mutagenic parameters such as sperm abnormalities and chromosomal aberrations indicate less/no mutagenic property of EFP in DL tumour-bearing mice.

Renal (renal function tests, RFT) and liver functions (liver functions test, LFT) are the crucial parameters for understanding the possible kidney and liver toxicity. Changes in serum creatinine and urea have been used to study renal function as important biomarkers [19]. In this study, mice cisplatin treatment resulted in an amplification of serum creatinine and urea levels, indicating the signs of nephrotoxicity. The increased creatinine and urea levels may be due to the reduced rate of glomerular filtration or increased ROS [41]. However, the EFP treatment did not indicate any notable changes in serum creatinine and urea levels, indicating that the EFP does not have any such nephrotoxicity as that observed for cisplatin. This is also supported by another report of earthworm extracts like powder of *Eudrillus euginae* and its toxicity evaluation in Wistar male rats, which suggested that the earthworm powder had no toxic effects in the kidneys in experimental animals [6].

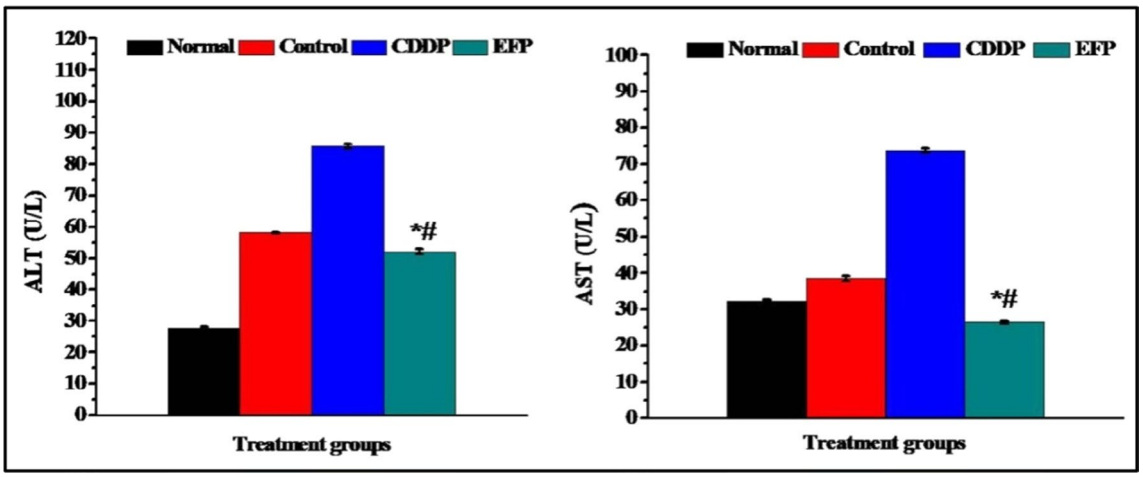


Fig. 15 Changes in serum ALT and AST in mice harbouring a tumour under various treatments. Results are expressed as mean SD ($n = 3$), *$P \leq 0.05$ compared to the control; # $P \leq 0.05$ compared to CDDP

Hepatic functions or LFT (liver function test) is generally determined with the assessment of certain marker enzymes like alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum of mice/humans under different experimental/pathological conditions [55]. In the present study, ALT and AST levels were elevated in cisplatin-treated mice, indicating hepatocellular injury in the liver. However, in EFP-treated mice, both AST and ALT levels did not illustrate any such elevation like cisplatin treatment, and it remained almost similar to control mice. This suggests less hepatocellular injury in the EFP-treated tumour-bearing mice. This is in agreement with other reports of hepatoprotective action of earthworm extract of *Lampito mauritii*, Kinberg was reported by Balamurgan et al., in 2008 against liver injury induced by paracetamol in Wister albino rat [6].

Conclusion

This is the first research study assessing the antitumour activity of earthworm extract (*Eisenia fetida* paste, EFP) from North-east India. *Eisenia fetida* paste was incredibly effective against murine ascites Dalton's lymphoma. EFP extract-mediated antitumour effect may potentially encompass the initiation of apoptosis and mitochondrial changes in tumour cells. A decrease in the GSH levels of DL cells after EFP treatment may reduce tumour cells protective ability, which may show more cytotoxic effects under these treatment conditions. Compared to the reference cisplatin, EFP has less/no mutagenicity, hepatotoxicity, and nephrotoxicity in the tumour-bearing hosts. Finally, it may be proposed that this study establishes foundational data for future laboratory investigations to discover novel biological compounds and advance drug development. This data may also help preserve biodiversity and strategically handle animal resources. Further studies may be initiated to isolate and characterise the bioactive composites from the earthworm extract and understand the molecular aspect of the antitumour mechanism.

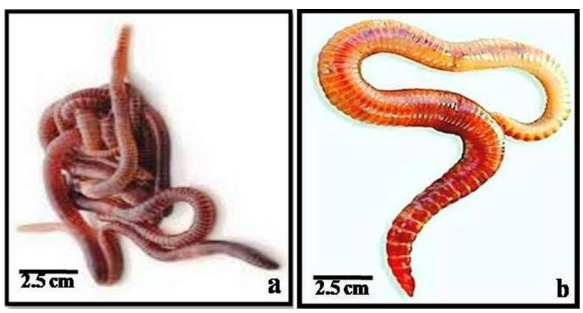


Fig. 16 *Eisenia fetida* (**a**. Live earthworm; **b**. Dead earthworm)

Materials and methods

An earthworm, *Eisenia fetida* (Fig. 16), was selected to assess the anticancer efficacy and toxicity in mice with ascites Dalton's lymphoma. The biochemical and toxicity assessment studies were also carried out and are presented in detail in the supplementary file. The earthworms were collected from the Jorhat and Dhemaji districts of Assam and identified with ZSI, Shillong's help. The earthworm extract /paste was prepared from the *E. fetida* to study antitumour activity.

Classification -

Kingdom: Animalia
Phylum: Annelida
Class: Clitellata
Order: Haplotaxida
Family: Lumbricidae
Genus: Eisenia
Species: *E. fetida*
Common name: Red worm (English), Kesu (Assamese)

Chemicals

Cisplatin (CDDP) solutions (1 mg/ml) were sourced from Biochem Pharmaceutical Industries in Mumbai, India; ethylenediaminetetra-acetic acid (EDTA), sodium arsenite ($NaAsO_2$), sodium sulphate (Na_2SO_4), analytical-grade 2-Thiobarbituric acid (TBA) and the required chemicals were employed in the study and purchased within the country. Refers and stains in double-glassed distilled water were always used to prepare the solutions.

Animal care and tumour maintenance

A total of 200 mice were used in the current study. Healthy, inbred Swiss albino mice of both sexes, aged 10–12 weeks and weighing 25–28 g, were procured from Pasteur Institute, Shillong, Meghalaya. An inbred mouse colony is being upheld within usual laboratory settings at 24 ± 2˚C. The mice are housed in propylene cages containing 5–6 animals each, and they are provided with food pellets available commercially (Amrut Laboratory, New Delhi) and unrestricted access to drinking water.

Experimental tumour model

The murine malignant tumour ascites Dalton's lymphoma (DL) was employed in the current study for assessing the antitumour activity and toxicity in the mice bearing tumour. The DL tumour was sustained in the laboratory through consecutive intraperitoneal (i.p.) injections of viable 1×10^7 tumour cells (0.25 ml

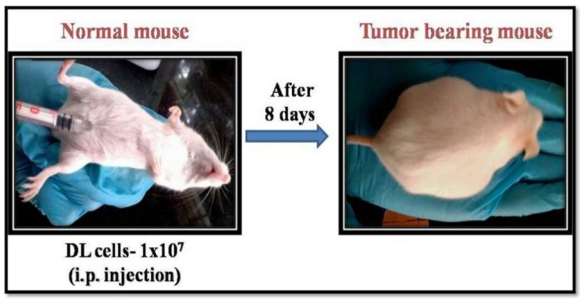


Fig. 17 Transplantation of ascites Dalton's lymphoma (DL) tumour cells in Swiss albino mice

volume in phosphate-buffered saline, PBS, pH 7.4) into healthy mice by the method described by [47] (Fig. 17). Typically, hosts receiving the tumour transplant survived for 19–21 days.

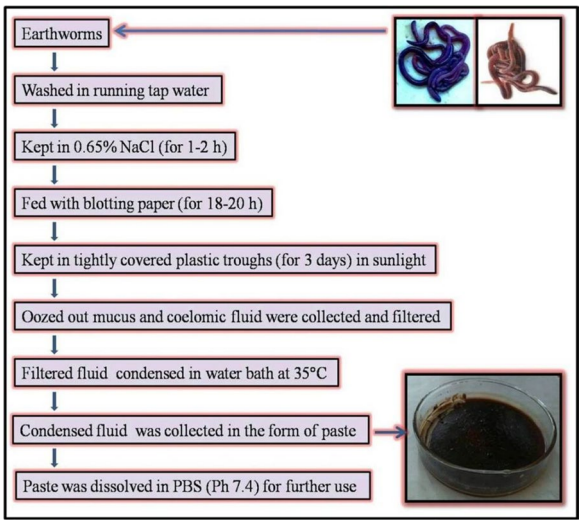


Fig. 18 Preparation of earthworm paste/extract

Preparation of selected earthworms' extract/paste

Fully matured earthworms, *E. fetida,* were collected from their natural habitats of Jorhat and Dhemaji district of Assam. The preparation of earthworm paste was done with the following technique with small modifications [5]. The earthworms were rinsed under flowing tap water to eliminate any sand particles present on the surface of earthworms and then kept in 0.65% NaCl for 1–2 h at room temperature; the earthworms were provided with moist blotting paper for a duration of 18 to 20 h to facilitate gut clearance. Subsequently, the worms with cleared guts were subjected to a distilled water rinse. These worms were placed in plastic troughs, securely covered with polythene, and exposed to sunlight for 3 days until completely dried. The mucus and coelomic fluid that exuded from the deceased worms caused the worms' digestion, creating a brown-hued earthworm paste (EFP). These earthworm pastes underwent filtration, and the acquired filtrates were concentrated using a water bath set at 35 °C. The obtained earthworm pastes were gathered and preserved at 4˚C until required for further procedures.

To screen anticancer potentials, the crude pastes/extracts were dissolved in phosphate buffer saline (pH 7.4) to acquire requisite dilution for the screening of antitumour activity (Fig. 18).

The per cent yield of the extracts was calculated with the following formula:

$$\%\ \text{yield of the extract} = \frac{\text{Weight of the extract (mg)}}{\text{Weight of the earthworm (mg)}} \times 100$$

The percentage yield of earthworm *Eisenia fetida* paste (EFP) was about 3.33%.

Treatment protocol and selection of specific dose for further treatment

Preliminary antitumour activities of the animal extracts were determined subsequently by the method of Sakagami et al. [50]. Dalton's lymphoma cells (1×10^7 cells in 0.25 ml PBS) were transplanted intraperitoneally (i.p.) into Swiss albino mice between 10 to 12 weeks old. The tumour transplantation day was considered as day '0'. Evidence of initial tumour growth became apparent within 3 to 4 days following tumour transplantation. Animals with tumour transplants were categorised into six groups for the two selected extracts, each consisting of 10 mice. Commencing on the 6th-day post-tumour transplantation, injections of *E. fetida* paste extracts (EFP) were administered via the intraperitoneal route once daily for five days. Group I, with control animals, was given 0.25 ml of the respective extract vehicle once daily for five days. Groups II, III, IV, V and VI were treated with 10, 20, 40, 80, and 160 mg/kg body weight/day of the earthworm's paste. Any instances of host mortality were documented daily, and the survival trends of the hosts were assessed across the various groups. The impacts of different doses on tumour inhibition were quantified as the percentage rise in average lifespan (ILS), with calculations conducted using the specified formula:

$$\text{ILS} = (\text{T/C x } 100) - 100$$

where T represents the mean survival days of the treated group of mice, and C denotes the mean survival days of the control group. To determine the most potent dose of the extract, animals were given a range of doses from

10 mg/kg of body weight to 160 mg/kg of body weight per day.

The tumour-transplanted mice of both sexes were allocated randomly into three experimental groups, with 10 mice in each group as follows:

Group-I: Tumour-bearing mice served as control and received PBS (Phosphate buffer solution) only.

Group- II: Tumour-bearing mice were administered with cisplatin daily for 5 consecutive days (i.p., 2 mg/kg body weight) beginning from the 6th day of post-tumour transplantation.

Group-III: Tumour-bearing mice were administered a therapeutic dose of EFP for five consecutive days (i.p., 40 mg/kg of body weight) beginning from the 6th day of post-tumour transplantation.

In different experiments, at intervals of 24, 48, 72, and 96 h following the final treatment (specifically on the 11th, 12th, and 14th days post-tumour transplantation), three mice from each group were euthanised using cervical dislocation [11]. DL cells, tissues (liver, kidney, testes) and blood samples were collected for microscopical, biochemical, and mutagenicity studies, as summarised in Fig. 19.

For conducting a comparative analysis of the antitumour effect, a recognised anticancer drug, cisplatin (CDDP), at a dose of 2 mg/kg body weight per day via intraperitoneal injection (i.p.), was employed as a reference compound. It was administrated to the host bearing the tumour on the 6th day for five consecutive days post-tumour transplantation. Cisplatin was employed as a reference drug by many researchers in the anticancer study as it has a unique ability to initiate apoptosis in numerous cancer cells [4].

The ascitic DL obtained from the mice was subjected to centrifugation at 2000xg and 4 °C for 15 min. The resulting pellet was then employed as the DL cells. The samples from the control and various treatment groups were used for microscopical, biochemical, enzymatic and haematological studies

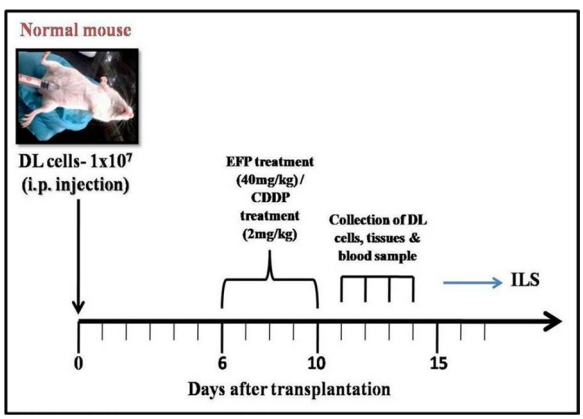


Fig. 19 Treatment schedule for survivability study of mice bearing ascites Dalton's lymphoma.

Cell viability study

The Trypan blue exclusion test was carried out to assess the viability of both DL cells and splenocytes. In brief, DL cells and splenocytes were harvested from DL-carrying mice in diverse groups at various time points (24, 48, and 96 h). Subsequently, they were washed twice with PBS. Following this, a portion of the cell mixture was combined with an equal quantity of Trypan blue dye (with 0.4% in PBS) and incubated for 2 min [53]. Unstained viable cells and stained non-viable or dead cells were quantified using a Neubauer haemocytometer mounted under a light microscope (Meiji). The percentage of non-viable cells was determined by observing 10–15 distinct selected fields of view within each treatment group, employing the subsequent formula:

$$\text{Percent dead cells} = \frac{\text{Average number of dead cells}}{\text{Average number of total cells}} \times 100$$

Fluorescence-based determination of apoptosis

Fluorescence-based apoptosis detection in DL cells was conducted using acridine orange and ethidium bromide (AO/EtBr) staining. DL cells were harvested from mice in various groups at distinct time intervals, precisely 24, 48, and 96 h, while subjected to diverse treatment conditions. Subsequently, they were washed with PBS. The cell suspension was supplemented with the AO/EtBr stain solution (100 µg/ml PBS of each dye), mixed gently, and then incubated for 5 min. The cells underwent a wash with PBS and were meticulously scrutinised under a fluorescence microscope (Leica) to identify any alterations or apoptotic features in the DL cells. Photographic documentation was also performed. A total of 1000 cells were screened, and the proportion of apoptotic and viable cells was tallied from twenty specifically chosen fields of view using a microscope. This procedure facilitated the determination of the apoptotic index.

Scanning electron microscopy and transmission electron microscopy of DL cells

Scanning electron microscopy (SEM)

Ascitic DL cells were collected from animals exposed to various experimental conditions. These cells were washed with PBS and subsequently resuspended in PBS, and a thin smear was prepared on a cover glass. A cover glass bearing the smear was then fixed in a 2.5%

(v/v) glutaraldehyde solution at 4°C. After fixation, the cells underwent rinsing in 0.1 M phosphate buffer and were steadily dehydrated using a sequence of ethanol concentrations: 30%, 50%, 70%, 90%, and 100%, each step lasting for 20 min. Next, the cover glass containing the cells was sectioned into smaller pieces, followed by critical point drying utilising a critical point dryer (CPD-030, BAL-TEC Co.). These dried samples were then fixed to an aluminium stub using double-stick tape, and a gold coating was applied using an ionic sputter coater (SCD-005, BAL-TEC Co.). The prepared samples were subsequently subjected to observation, meticulous examination, and imaging using a Scanning Electron Microscope (JEOL JSM – 6360).

Transmission electron microscopy (TEM)

The DL tumour was gathered from mice within distinct experimental groups and centrifuged at 1000xg for 10 min at 4 °C. The resulting cell pellets were washed in PBS and then fixed in 3% glutaraldehyde at 4 °C for 2 h. After fixation, they were rinsed in 0.1 M cacodylate buffer. The cell pellets were fragmented into smaller fragments, followed by post-fixation in 1% osmium tetroxide at 4 °C for 15 min. Subsequently, a graded series of acetone (30–50-70–80-90–95%, with two repetitions at each concentration for 15 min each), was used for dehydration. The cell pellets were then placed in dry acetone prepared by adding an excess of CuSO4 crystals to absolute acetone and filtering.

Further steps included two rounds of propylene oxide treatment for 1 h at room temperature, followed by a mixture of propylene oxide and an embedding medium composed of Araldite Cy212, dodecenyl succinic anhydride, tridimethylamino methyl phenol, and dibutylphdyhalate. Thin sections (60–80 nm) were sliced using an ultramicrotome (ultratome-RMC, MTX, USA) and collected on copper grids. These sections were stained using a mixture of lead citrate (5%) and uranyl acetate (5%) (1:1; v/v). The examination used an electron microscope (Jeol electron microscope) operating at 80 kV. The sections were scanned and meticulously inspected, and photomicrographs were captured.

Mutagenic studies

Sperm abnormality studies

On the fifth day of treatment, male mice within various groups were euthanised. The cauda epididymis was excised and immersed in physiological saline. Subsequently, the cauda epididymis was minced into smaller fragments and allowed to rest undisturbed for 20 min to enable the dispersal of spermatozoa. The spermatozoa were then dispersed onto a dirt-free slide, air-dried, and subjected to fixation in absolute methanol for 15 min. On the subsequent day, the spermatozoa were stained using a 1% aqueous solution of eosin-Y. Five hundred spermatozoa were examined for each mouse to identify aberrations in both tail shapes and sperm heads. The assessment followed the criteria established by Wyrobek and Bruce in 1975 [27].

Analysis of chromosomal aberration

An analysis of the chromosomal aberration was conducted according to a methodology outlined in 1994 by Sharma and Sharma [28]. Mice within different groups underwent mitotic arrest, initiated 2 h before euthanasia through an intraperitoneal injection of colchicine (4 mg/kg body weight). The collection of bone marrow cells was done by centrifugation at 1000 rpm and 4 °C for 5 min. The bone marrow was extracted from the femur through continuous flush with PBS (pH 7.4) with a syringe. Following this, the cell pellet was introduced to a pre-warmed hypotonic solution (1% sodium citrate) of 5 mL, and incubation was carried out for 20 min at 37 °C. Subsequently, Carnoy's fixative (methanol: glacial acetic acid, 3:1, v/v) in drops were gently mixed, and the mixture was centrifuged for 5 min at 1000 rpm. The upper layer was removed, and the resulting pellets were gradually separated by gentle tapping. Chilled Carnoy's fixative (5 mL) was introduced to the mixture and incubated for 30 min at 4 °C. Two more repetitions of the fixation process were carried out with a 30-min break. Ultimately, the cells were resuspended in 0.5 mL of the fixative. A small amount of drops of this suspension was placed on dirt and grease-free chilled slides, followed by flaming to facilitate drying. Once air-dried, the slides were stained the following day with Giemsa stain solution (freshly prepared) in Sorensen's buffer (pH 6.8). Subsequently, the slides were mounted using DPX. A hundred well-spread metaphase cells were examined per animal under a light microscope (Leitz). Various chromosomal abnormalities were identified and scored based on observation.

Renal function (RFT) and liver function (LFT) tests

Blood samples were obtained from mice within distinct experimental groups on the fifth day after treatment through orbital venous sinus bleeding. Two millilitres of blood collected from the mice through orbital venous sinus bleeding and serum was separated by centrifuged at 4000 rpm for 10 min. The collected blood was then processed to separate the serum. After serum separation, changes in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were evaluated to gauge liver function. Additionally, serum urea and creatinine levels were measured to assess renal function. This assessment was conducted using a Clinical Chemistry Analyzer (SYNERGY BIO-1904C).

Statistical analysis

All experimental data were presented as the mean ± standard deviation (S.D.), with each determination repeated three times. The differences among multiple groups were assessed using a one-way analysis of variance (ANOVA), followed by a post hoc test (Tukey test). A significance level of $P \leq 0.05$ was deemed as statistically significant. Data were analysed using Origin 8 Software.

Abbreviations

DL	Dalton's lymphoma
EFP	*Eisenia fetida* Paste
CT	Computed tomography
MRI	Magnetic resonance imaging
PET	Positron emission tomography
SD	Standard deviation
CDDP	Cis-diamminedichloroplatinum(II)
ILS	Increase in life span
AO/EtBr	Acridine orange/ethidium bromide
BSH	Banana-shaped head
BLH	Balloon-like head
HSH	Hammer-shaped head
CN	Coiled neck
BH	Bulged head
ANOVA	Analysis of variance
ICB	Isochromatid break
CF	Chromosomal fragment
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
SGPT	Serum glutamic pyruvic transaminase
SGOT	Serum glutamic-oxaloacetic transaminase
DNA	Deoxyribonucleic acid
AMPK	AMP-activated protein kinase
RFT	Renal function test
LFT	Liver function test
ROS	Reactive oxygen species
GSH	Glutathione

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Author contributions

Both the authors are equally involved in conceptualising this research work. Necessary data/sample collection, laboratory experiments, preparation of graphs, tables, Statistical analysis, interpretation and initial manuscript preparation were done by MPB. SBP did further correction and scrutiny of the manuscript. We take the responsibility for the integrity of the work presented in this manuscript. All the authors have read and approved the final manuscript.

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Availability of data and materials

All the data that support this study are included in the manuscript.

Declarations

Ethics approval and consent to participate

All applicable international, national, and institutional guidelines for the care and use of animals were followed. The maintenance, use of the animals and the experimental protocol of the present study were approved by the North-Eastern Hill University, Shillong, Meghalaya, India, vide PhD registration no. 2247 of 2013 and Institutional Ethics Committee (animal models), North-Eastern Hill University, Shillong, India, dated 21–11-2014.

Consent for publication

All the mentioned authors agreed to submit the work to the Future Journal of Pharmaceutical Sciences.

Competing interests

The authors declare no conflict of interest.

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