In vitro Antiproliferative Effect of Earthworm Coelomic Fluid of Eudrilus Eugeniae, Eisenia foetida, and Perionyx Excavatus on Squamous Cell Carcinoma-9 Cell Line: A Pilot Study

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INTRODUCTION

The specific problem encountered in combating cancer is the uncontrolled proliferation of cancer cells and metastasis which is a multistep complex event during the growth of malignant tumors. It is influenced by inherent properties of tumor proper, systemic, and local environmental host factors. Natural products have been regarded as important sources that could produce potential chemotherapeutic agents. Over 50% of anticancer drugs approved by the United States Food and Drug Administration since 1960 were originated from the natural resources. The earthworms are complex invertebrates which synthesize a variety of immunoprotective molecules and produce several types of leukocytes. They possess innate immunity, as well as some functions associated with the adaptive immunity (allogeneic tissue rejection). These molecules exhibit different activities, such as fibrinolytic, anticoagulative, anticancer, antimicrobial, and thus may be exploited for the treatment of variety of diseases.

Recently, concepts of using naturally available exudates from earthworms to inhibit proliferation of cancer cells have emerged. Few studies on

Abbreviations Used:
- ECF: Earthworm coelomic fluid
- EE: Eudrilus Eugeniae
- EF: Eisenia foetida
- PE: Perionyx Excavatus
- SCC: Squamous cell carcinoma
- BSA: Bovine serum albumin
- PBS: Phosphorized buffered saline
- MTG: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
- IC50: Inhibitory concentration

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the breast, liver, and brain tumors have been employed with limitations, but to the best of our knowledge yet to be explored in oral cancer, this necessitates the need for this study.

The previous studies that explored the antiproliferative potentials of earthworm coelomic fluid (ECF) have evaluated one species at a time; the current study has distinctively compared antiproliferative efficacy of three species of earthworms simultaneously under standard clinical settings on oral cancer cell line squamous cell carcinoma (SCC)-9. Earthworm species such as PE has also been explored in this study which has not been reported earlier.

The aim of the present study is to explore the antiproliferative effect of ECF of three identified species of Eudrilus eugeniae (EE), Eisenia fetida (EF), and Perionyx excavatus (PE) on oral cancer cell line SCC-9. The results obtained would pave the way for subsequent exploration in this field of research.

MATERIALS AND METHODS

Collection of coelomic fluid

Ethical approval was obtained from the University ethics committee. Earthworms were procured from a local vermicomposting farm where grouping of species was done before use. A zoologist identified and authenticated the organisms used in this study with the reference features. A zoologist identified and authenticated the organisms used in this study with the reference features. A zoologist identified and authenticated the organisms used in this study with the reference features. A zoologist identified and authenticated the organisms used in this study with the reference features. A zoologist identified and authenticated the organisms used in this study with the reference features. A zoologist identified and authenticated the organisms used in this study with the reference features. A zoologist identified and authenticated the organisms used in this study with the reference features. A zoologist identified and authenticated the organisms used in this study with the reference features.

Protein estimation by modified Bradford method

The concentration of protein was estimated using the modified Bradford assay. Bovine serum albumin (BSA) 4 mg/ml, dissolved in phosphed buffered saline (PBS), was used as standard. Briefly, 10 μL of each protein sample and BSA standards were mixed with 250 μl Bradford reagent (Sigma Aldrich). The absorbance at 595 nm of each sample mixture, that is, proportional to the quantity of solubilized protein was measured using a Tecan plate reader and the values were plotted.

Protein estimation by Biuret method (method of validation)

Duplicates of 6 mg/ml of BSA were pipetted. Volume of distilled water was adjusted to 1 ml for the blank. About 2 ml of Biuret reagent mixed and incubated at 37°C for 20 min. Optical density at 550 nm was recorded using spectrophotometer.

A calibration curve was constructed by plotting average optical density reading on “Y” axis against standard protein concentration (in mg) on “X” axis. Value “X” was recorded from the graph corresponding to the optical density reading for the test.

Antiproliferative 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide assay

The cell line employed in the present study was SCC-9 (Origin: Homo sapiens – Tongue tissue) procured from American Type Culture Collection (ATCC). The oral cancer cell lines (SCC-9 cells) were grown in minimal essential medium supplemented with 4.5 g/L glucose, 2 mmol/L L-glutamine, and 5% fetal bovine serum (growth medium) at 37°C in 5% CO₂ incubator. SCC-9 were seeded in a 96-well plate at a concentration of 50,000 cells/well and incubated for 24 h at 37°C, 5% CO₂ incubator.

The cells were treated with different concentrations of test compounds (2.5, 5, 10, 20, 40, and 80 μg/ml) of coelomic fluid of the three test species, respectively, for 24 h. Colchicine was taken as positive control and saline as negative control.

After 24 h incubation with test samples, 100 μl/well of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent at concentration of 5 mg/10 ml in 1% PBS was added to the respective wells and incubated for 3–4 h. After incubation with MTT reagent, it was discarded by pipetting without disturbing the cells. About 100 μl of dimethyl sulfoxide was added to rapidly solubilize the formazan. The optical density (OD) was measured at 590 nm. The effective lethal concentration required for antiproliferative effect was determined by plotting a graph and obtaining a curve with maximum number of cells killed and concentration of the coelomic fluid used. The percentage inhibition was calculated using the formula: (OD of control – OD of sample/OD of control) × 100. The inhibitory concentration or (IC₅₀) (drug concentration that is required to reduce half of the cells from the total population) was ascertained using GraphPad Prism 7 software (San Diego, California). Chi-square test was used to analyze the antiproliferative efficacy between samples.

RESULTS

Collection of coelomic fluid

Cold-shock method of fluid collection was found to be the safest method of coelomic fluid collection. This method of placing worms under the ice was least harmful as seen by the survivability of the worms after each time of collection as shown in Figure 1. About 3.5 ml of ECF was obtained from EE, 3 ml from EF, and PE, respectively. The fluid collected was centrifuged and stored at –80°C. The survivability of the worms was appreciable even after three rounds of fluid collection.

Protein estimation by modified Bradford method

The Modified Bradford protein assay for estimation of total protein concentration was preferred over the Lowry method as it is simpler, faster, and more sensitive. It is subjected to less interference by common reagents and nonprotein components of biological samples. The total protein values obtained for earthworm species EE, EF, and PE were 2.37, 1.94, and 3.41 mg/ml, respectively, as shown in Table 1.

Protein estimation by Biuret method (method of validation)

The results obtained by the Bradford protein assay method was validated using the biuret protein estimation method, the protein values obtained were similar for the three earthworm species as shown in Table 2.

Antiproliferative 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide assay

ECF of EE, EF, and PE at concentrations of 2.5, 5, 10, 20, 40, and 80 μg/ml on SCC-9 cells showed significant dose-dependent inhibition of growth of SCC-9 cells at IC₅₀ values of 4.6, 44.69, and 5.27 μg/ml, respectively, as shown in Table 3. GraphPad Prism 7 software was used to determine the IC₅₀ values as shown in Figure 3. Positive control drug colchicine exhibited an IC₅₀ value of 11.90 μg/ml as shown in Table 4 and Figure 4.
Table 1: Protein concentration of samples (Bradford method)

| Samples | OD at 595 nm | Concentration in mg/ml |
|---------|-------------|------------------------|
| EE-1    | 1.19        | 2.37                   |
| EF-2    | 1.05        | 1.94                   |
| PE-3    | 1.53        | 3.41                   |

OD: Optical density; EE: Eudrilus Eugeniae; EF: Eisenia fetida; PE: Perionyx excavatus

Table 2: Protein concentration of samples (Biuret method)

| Samples | OD at 595 nm | Concentration in mg/ml |
|---------|-------------|------------------------|
| EE-1    | 0.116       | 2.40                   |
| EF-2    | 0.106       | 1.90                   |
| PE-3    | 0.136       | 3.41                   |

OD: Optical density; EE: Eudrilus Eugeniae; EF: Eisenia fetida; PE: Perionyx excavatus

Table 3: Results of 3-(4, 5-Dimethylthiazol-2-Yl)-2, 5-Diphenyltetrazolium Bromide assay-Coelomic fluid of the three species and the percentage inhibition obtained

| Earthworm species | Concentration (µg/ml) | Absorbance at 590 nm | Percentage inhibition | IC₅₀ |
|-------------------|-----------------------|----------------------|-----------------------|-----|
| Control           | 0.0                   | 0.562                | 0.00                  |     |
| EE-1              | 2.5                   | 0.478                | 14.93                 | 4.60 µg/ml |
|                   | 5                     | 0.401                | 28.63                 |     |
|                   | 10                    | 0.254                | 54.80                 |     |
|                   | 20                    | 0.199                | 64.58                 |     |
|                   | 40                    | 0.157                | 72.06                 |     |
|                   | 80                    | 0.109                | 80.60                 |     |
| EF-2              | 2.5                   | 0.556                | 1.00                  | 44.69 µg/ml |
|                   | 5                     | 0.520                | 7.46                  |     |
|                   | 10                    | 0.437                | 22.16                 |     |
|                   | 20                    | 0.404                | 28.17                 |     |
|                   | 40                    | 0.215                | 61.70                 |     |
|                   | 80                    | 0.128                | 77.26                 |     |
| PE-3              | 2.5                   | 0.457                | 18.67                 | 5.27 µg/ml |
|                   | 5                     | 0.399                | 28.99                 |     |
|                   | 10                    | 0.299                | 46.79                 |     |
|                   | 20                    | 0.236                | 54.44                 |     |
|                   | 40                    | 0.201                | 64.23                 |     |
|                   | 80                    | 0.188                | 66.54                 |     |

IC₅₀: Inhibitory concentration; EE: Eudrilus Eugeniae; EF: Eisenia fetida; PE: Perionyx excavatus

Chi-square test showed difference in efficacy of antiproliferative effect between samples. EE and PE showed highly significant difference compared to EF. The difference in efficacy of antiproliferative effect between EE and PE was insignificant [Table 5].

DISCUSSION

Oral cancer is the sixth most common cancer in males and the twelfth most common in females. In developing countries, such as India, it is the most common cancer. Approximately, 94% of all oral malignancies are squamous cell carcinoma.[29] Over the past few decades, researchers have explored alternate therapies and remedies to prevent its progression but have succumbed to low success rates. Chemotherapy plays as a double-edged sword; apart from killing cancer cells it also kills certain adult cells that divide more rapidly, such as gastrointestinal lining, bone marrow cells, and hair follicles, thereby causing significant adverse effects. Targeted therapy of oral cancer is promising following identification of anticancer biomolecules.[26] Natural ways to prevent cancer recurrence is currently the latest trend in cancer therapeutics.

Naturally available extracts have been sought after in this regard as an adjunctive therapeutic modality.[21] Current research in the head and neck cancer mainly focuses to understand the molecular mechanisms of oral cancer development and progression to target the biomarkers and facilitate the development of new treatment strategies.[22] Studies with cell lines can serve as an initial screen for agents that might regulate drug resistance and to establish whether the differences exist in the different drug-resistant sublines.[23,24]

In the present study, we used tongue cancer cell line SCC-9 from ATCC to perform the cytotoxic study. Veeramani studied the characterization of coelomic fluid of EE and demonstrated that the cold shock method is a reliable technique for collection of ECF.[25] In this study, cold-shock method of fluid collection was employed to collect 3.5 ml from species EE, 3 ml from EF, and 3 ml from PE, respectively. In the cold-shock method, the earthworms secrete comparatively larger volume of fluid (1.5 ml) than other methods. The fluid collected is clear brown without any debris.

The Bradford protein assay employs the principle of Coomassie Blue G250 dye binding to protein.[11] The Biuret test which uses complexation of copper ions to functional groups in the protein’s peptide bonds was employed to validate the protein analysis results obtained from the modified Bradford protein assay.[21]

This accurate protein estimation test has been employed in few studies like the one performed by Merzouk et al. to estimate the total proteins in Leech saliva extract.[28] Traditionally, in vitro determinations of toxic effects of unknown compounds have been performed by counting viable cells after staining with a vital dye.

Alternative methods used are measurement of radioisotope incorporation as a measure of DNA synthesis, counting by automated counters, and others which rely on dyes and cellular activity.
Table 4: Percentage inhibition of colchicine (positive standard) on squamous cell carcinoma-9 cells

| Compound name | Concentration μM | OD at 540 nm | Percentage inhibition | IC50 |
|---------------|------------------|--------------|-----------------------|------|
| Colchicine    | Control          | 0.5926       | 0.00                  |      |
|               | 1.57             | 0.5609       | 5.35                  | 11.9 |
|               | 3.125            | 0.5012       | 15.42                 |      |
|               | 6.25             | 0.4181       | 29.45                 |      |
|               | 12.5             | 0.3811       | 35.69                 |      |
|               | 25               | 0.3351       | 40.42                 |      |
|               | 50               | 0.2911       | 50.88                 |      |
|               | 100              | 0.2051       | 65.39                 |      |

OD: Optical density; IC50: Inhibitory concentration

Table 5: Results of Chi-square analysis

| Test sample          | χ²       | P<0.05 - significant | Inference |
|----------------------|----------|----------------------|-----------|
| EE versus EF         | 28.031   | 0.000003589          | Significant |
| EF versus PE         | 33.526   | 0.00000296           | Significant |
| EE versus PE         | 1.543    | 0.98085492           | Not significant |

EE: Eisenia fetida; EF: Eisenia fetida; PE: Perionyx excavatus

The MTT assay is a means of measuring the activity of living cells through mitochondrial dehydrogenases.[32,33] The resulting purple solution is spectrophotometrically measured.[34,35] An increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of cytotoxicity caused by the test.

In the present study, ECF of EE, EF, and PE was used at concentrations of 2.5, 5, 10, 20, 40, and 80 μg/ml on SCC-9 cells in vitro and evaluated for antiproliferative efficacy by MTT assay. Colchicine, a standard anticancer drug was used as a positive control.

The test samples EE, EF, and PE treatment showed significant dose-dependent inhibition of growth of SCC9 cells at IC50 values of 4.6, 44.69, and 5.27 μg/ml, respectively. Positive control drug colchicine exhibited an IC50 value of 11.90 μg/ml. These results suggest that all three test samples have antiproliferative effect against SCC-9 cells [Figure 5].

In the earlier studies, ECF was employed to demonstrate antiproliferative activity on other types of cancers. XIE Jiang Bi et al. in 2003 studied the in vitro antitumor activity of the earthworm EF on HT-116, SY5Y, K562, MGe803, and HeLa cell lines and 50% of growth inhibition was observed at 60-110 mg/L of dose. The authors also reported on in vivo results of the prolonged lifespan of ascites tumor (S180) bearing mice. [34] HE Dao-wei in 2005 performed an in vitro study to evaluate the inhibitory effects of earthworm extract on the cellular growth of Eca-109. The results demonstrated a dosage of (900,450 mg/L) had prominent inhibitive effects on Eca-109 cells.[32]

The antitumor activity of EFE (earthworm fibrinolytic enzyme), isolated from EF, on human hepatoma cells in vitro and in vivo was evaluated by Chen et al. in 2007. A dose-dependent in vitro inhibition was observed. The growth of tumor in nude mice was significantly suppressed in EFE group compared to the control group.[35] The cytotoxic and apoptotic activity of the EF coelomic fluid was evaluated in vitro by Yanqin et al. in 2007. A concentration of 1 mg/ml exhibited inhibitory effects on HeLa cells with an inhibition rate of 84.22%.[34]

Mohamed Jaibir et al. in 2011 tested anticancer activity of the coelomic fluid of the earthworm EE in SiHa cells in vitro. At higher concentrations of 80 μg/ml, the cell death observed was 68% and at 100 μg/ml, the cell death was 89%. The IC50 concentration was determined to be 50 μg/ml.[36] Dinesh et al. in 2013 evaluated the cytotoxic effect of coelomic fluid from EE on HeLa cells, colon cancer cells, leukemic cells, and brain tumor cells in vitro and found a dose-dependent inhibitory effect.[36]

Antitumor activity of serine protease from the Indian earthworm Pheretima posthuma on MCF-7 cells was determined by Verma et al. 2013. An inhibition of 38.5% at concentration of 276.04 μg/ml and 263.14 μg/ml was observed.[37] In vitro anticancer activity of the earthworm powder (EWP) obtained from Lampito mauritii in HT-29 cells was evaluated by Lourdowny and Ramesh in 2014. At low dilution rates (10 μg/ml), the viability was unaffected; however, at higher concentration (320 μg/ml) 82% growth inhibition or cytotoxicity was observed.[38] Only meager studies on the effect of ECF of species EE, EF, and PE on cancer cell lines have been performed in India, and to the best of our knowledge, globally, none have collectively evaluated ECF of three species. There is limited information available on the effect of ECF on oral cancer cells which is scarcely researched area in oncology and appears to have not been attempted.

Anticancer properties of earthworms species such as PE which was yet to be explored on any type of cancer has shown to have promising antiproliferative effect on oral cancer cells SSC-9 (IC50=5.27 μg/ml) along with earthworm species EE and EF that showed an IC50 values of 4.6 μg/ml and 44.69 μg/ml respectively.

Testing in cancer cell lines has remained the initial step for drug testing for many years. It is thereby considered the first step in assessing several complex therapeutic preparations before its use in large scale in vivo.
Cytotoxicity evaluation in cancer cell lines has been advantageous and is expected to provide results which extrapolate with the original tumor. [39] There has been an increasing interest to research natural products available in nature, which can combat cancer and its side effects, and prevent them from occurring and increase the lifespan and quality of life of patients. The present study has shown ECF of EE, EF, and PE has an appreciable antiproliferative effect on oral cancer cell line SCC-9, with EE showing the best effect followed by PE and EF. The antiproliferative effect was variable among the three species.

Targeted therapies developed from cell lines in vitro may be translated in vivo directed against the primary tumor at the cellular level of tumor development, and thus, this therapy may find its way in the treatment of early-stage head and neck cancer. [24, 40] There is certainly scope to translate these findings in clinical settings.

CONCLUSION

The results obtained in this study revealed that ECF of EE, EF, and PE has antiproliferative potential on SCC-9 cells with following IC50 values 4.6, 5.27 and 44.69 μg/ml for EE, PE, and EF, respectively, and could be useful for the development of novel therapeutic agent against oral cancer with negligible side effects. The limitations of this study include use of a single cancer cell line to explore the antiproliferative potential. However, the uniqueness of the present study is that the antiproliferative potential of 3 earthworm species has been compared together. EE and EF have known antiproliferative effect on other cancer cell lines, we have demonstrated on oral cancer cell lines. The antiproliferative potential of earthworm species PE has not been explored thus far. It can be concluded that the ECF of EE and PE are more efficacious than EF comparatively based on the IC50 determined. The scope and future avenues are to ascertain the specific bioactive molecules responsible for this antiproliferative activity, perform higher anticancer experiments, and determine ECF mechanism of action on cancer cells. These experiments are ongoing in our laboratory. These bioactive molecules need to be screened against different cell lines apart from the selected cell line used to ensure the wide range of their antiproliferative action. Antiproliferative effect of ECF of different species of earthworm obtained from this study is promising and necessitates performance of advanced anticancer studies on oral cancer cell lines.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Lu P, Weaver VM, Werb Z. The extracellular matrix: A dynamic niche in cancer progression. J Cell Biol 2012;196:395–406.
2. Kim J, Park EJ. Cytotoxic anticancer candidates from natural resources. Curr Med Chem Anticancer Agents 2002;2:485-537.
3. Cooper EL, Balamurugan M, Huang C Y, Tsao CR, Heredia J, Tommaseo-Ponzetta M, et al. Earthworms diel: Ancient, inexpensive, noncontroversial models may help clarify approaches to integrated medicine emphasizing neuroimmune systems. Evid Based Complement Alternat Med 2012;2012:164152.
4. Cooper EL, Cossarizza A, Kauschke E, Franceschi C. Cell adhesion and the immune system: A case study using earthworms. Microcos Res Tech 1999;44:237-53.
5. Cooper EL, Hrzenjak TM, Girdsa M. Alternative sources of fibroinolitic, anticoagulative, antimicrobial and anticancer molecules. Int J Immunopathol Pharmacol 2004;17:237-44.
6. Jagtap S, Meganathan K, Wagh V, Winkler J, Hescheler J, Sachindia A, et al. Chemoprotective mechanism of the natural compounds, epigallocatechin-3-O-gallate, quercetin and curcumin against cancer and cardiovascular diseases. Curr Med Chem 2009;16:1451-62.
7. Ansari AA, Saywack P. Identification and classification of earthworm species in Guyana. Int J Zool Res 2011;7:93-9.
8. Cynthia JM, Uma K, Uma Devi R. Bacteriostatic effect of Lampitio mauritii (Kinberg) coelomic fluid and cell extract on pathogens. Int J Curr Microbiol App Sci 2014;3:182-6.
9. Packia Lekshmi NC, Viveka S, Sahila Kumari R, Selva Bharath M, Jeeva S, Rajabirutha J, et al. Synthesis of nanofibre and silver nanoparticles from coelomic fluid of earthworm, eudrilus eugeniae and ponto scolex corethrurus and its antimicrobial potency. Asian J Pharm Clin Res 2014;7:177-82.
10. Eyambe G, Goven AJ, Fitzpatrick LC, Cooper KL. Extrusion protocol for use in chronic immunotoxicity studies with earthworm’s coelomic fluid. Lab Anim 1991;25:61-7.
11. Kruger NJ. The Bradford method for protein quantitation. In: Walker JM, editor. The Protein Protocols Handbook. New Jersey: Humana Press; 2002. p. 15-21.
12. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-54.
13. Compton SJ, Jones CG. Mechanism of dye response and interference in the Bradford protein assay. Anal Biochem 1985;151:369-74.
14. Guobing X, Lili J, Lihua Z, Tien X. Application of an improved biuret method to the determination of total protein in urine and cerebrospinal fluid without concentration step by use of Hitachi 7170 auto-analyzer. J Clin Lab Anal 2001;15:161-4.
15. Lundin A, Hassenson M, Persson J, Pousette A. Estimation of biomass in growing cell lines by adenosine triphosphate assay. Methods Enzymol 1986;133:27-42.
16. Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB. Evaluation of a tetrazolium-based semiautomated colorimetric assay: Assessment of chemosensitivity testing. Cancer Res 1987;47:936-42.
17. Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods 1986;89:271-7.
18. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55-63.
19. Bhargava A, Saigal S, Chalishazar M. Histopathological grading systems in oral squamous cell carcinoma: A review. J Int Oral Health 2010;2:1-10.
20. Tanaka T, Ishigamori R. Understanding carcinogenesis for fighting oral cancer. J Oncol 2011;2011:603740.
21. Danaraddi S, Koneru A, Hunasgi S, Ramalu S, Vanishree M. Natural ways to prevent and treat oral cancer. J Oral Res Rev 2014;6:34-9.
22. Wilding JL, Bodmer WF. Cancer cell lines for drug discovery and development. Cancer Res 2014;74:2377-84.
23. Ferreira D, Adega F, Chaves R. The importance of cancer cell lines as in vitro models in cancer methymelone analysis and anticancer drugs testing. Oncogeneics Cancer Proteomics 2013;8:139-65.
24. van Staveren WC, Solis DY, Hebrant A, Detours V, Dumont JE, Maenhaut C, et al. Human cancer cell lines: Experimental models for cancers in situ? For cancer stem cells? Biochim Biophys Acta 2009;1795:92-103.
25. Veeramani A. Study on the Characterization of Coelomic Fluid of Eudrilus Eugeniae and its Effect on Plants and Animal Cells in 19th in-vitro culture system. Bharathidasan University; 2010. Available from: http://www.shodhganga.inflibnet.ac.in/handle/10603/9491. [Last accessed on 2016 Nov 1].
26. Merzouk A, Ghawi AM, Abdualkader A, Abdullahi AD, Alaama M. Anticancer effects of medical Malaysian Leech Saliva Extract (LSE). Pharm Anal Acta 2012;15:1-5.
27. Crouch SP, Kozloowski R, Slater KJ, Fletcher J. The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. J Immunol Methods 1993;160:81-8.
28. Gonzalez RJ, Tarloff JB. Evaluation of hepatic subcellular fractions for Alamar blue and MTT reductase activity. Toxicol In Vitro 2001;15:257-9.
29. Hattori N, Sakakibara T, Kajiyama N, Igarashi T, Maeda M, Murakami S, et al. Enhanced microbial biomass assay using mutant luciferase resistant to Benzalkonium chloride. Anal Biochem 2003;319:287-95.
30. Kangas L, Grönroos M, Nieminen AL. Bioluminescence of cellular ATP: A new method for evaluating cytotoxic agents in vitro. Med Biol 1984;62:338-43.
31. Jiang B, Wei Guo H, Ning W, Zhen Quan G, Mei Min Y, Ning L, et al. Extraction and isolation of the anti-tumor protein components from earthworm (Eisenia fetida andrei) and the anti-tumor activity. Chin J Biochem Mol Biol 2003;19:359-66.
32. He DW, Zhou F. Study on the anti-tumor effects of earthworm extract. J Yangtze Univ (Nat Sci Edit) (Chin) 2005;18:225-8.
33. Chen H, Takahashi S, Imamura M, Okutani E, Zhang ZG, Chayama K, et al. Earthworm fibrinolytic enzyme: Anti-tumor activity on human hepatoma cells in vitro and in vivo. Chin Med J (Engl) 2007;120:898-904.
34. Yangin L, Yan S, Zhenjun S, Shijie L, Chong W, Yan L, et al. Coelomic fluid of the earthworm Eisenia fetida induces apoptosis of HeLa cells in vitro. J Eur Soil Biol 2007;43:143-8.
35. Mohamed Jaabir MS, Shamsheerali L, Yasir M, Senthil Kumar S. Evaluation of the cell-free coelomic fluid of the earthworm Eudrilus Eugeniae to induce apoptosis in SiHa cell line. J Pharm Res 2011;4:3417-20.
36. Dinesh MS, Sridhar S, Chandana PG, Pai V, Geetha KS, Naveen Hegde R. Anticancer potentials of peptides of coelomic fluid of earthworm Eudrilus Eugeniae. Biosci Biotechnol Res Asia 2013;10:601-6.
37. Verma MK, Xavier F, Verma YK, Sobha K. Evaluations of cytotoxic and anti-tumor activity of partially purified serine protease isolate from the Indian earthworm Pheretima posthuma. Asian Pac J Trop Biomed 2013;3:896-9.
38. Lourdumary AJ, Ramesh N. Evaluation of anticancer activity of Indian Earthworm Lampito mauritii. Int J Res Pharm Sci 2014;4:27-30.
39. Lin CJ, Grandis JR, Carey TE, Gollin SM, Whiteside TL, Koch WM, et al. Head and neck squamous cell carcinoma cell lines: Established models and rationale for selection. Head Neck 2007;29:163-88.
40. McDermott M, Eustace AJ, Busschots S, Breen L, Crown J, Clynes M, et al. In vitro development of chemotherapy and targeted therapy drug-resistant cancer cell lines: A Practical guide with case studies. Front Oncol 2014;4:40.