Earthworm, a novel in vivo system to validate antimitotic compounds

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Abstract: We report here that earthworm can be used as an in vivo system to prescreen antimitotic compounds. The known antimitotic compound colchicine and the aqueous extract of Acorus calamus L. rhizome were used to examine this system. The antimitotic activity of colchicine and the rhizome extract was confirmed by methods with Allium cepa root tip, earthworm regeneration, and MTT (3-(4,5-dimethyl thiazolyl-2-yl)-2,5-diphenyltetrazolium bromide). An earthworm regeneration assay examined the regeneration ability of tissues from amputated regions of the earthworm. All 3 assays showed that cell division is inhibited with colchicine and the aqueous extract of A. calamus rhizome. Histology studies with amputated earthworms confirmed that the development of the blastema was observed only in control worms. Immunohistochemistry analysis with proliferation cell nuclear antigen and phospho serine 10 histone H3 antibodies showed that amputated worms treated with colchicine or the aqueous extract of A. calamus did not have active cell division. MTT assay with the MCF-7 cell line (human breast carcinoma) further confirmed that the aqueous extract of rhizome affected cell proliferation. Our results suggest that the earthworm could be used to prescreen the antimitotic potential of plant extracts or other unknown compounds. It is simple, reproducible, and cost-efficient compared to animal cell line-based methods.

Key words: Antimitotic, Acorus calamus, colchicine, earthworm regeneration assay, Eudrilus eugeniae, immunohistochemistry

1. Introduction
Antimitotic compounds identified from plants have shown great success in the treatment of cancer (Gascoigne et al., 2009; Weaver, 2014). Research on the screening of anticancerous compounds has been increasing due to the availability of millions of natural resources (Fouché et al., 2008; Fadeyi et al., 2013). To evaluate the antimitotic potential of an unknown compound, plant-based methods using onion root tips, potato tubers, or green gram seeds have been in practice (Eigsti, 1938; Coker et al., 2003; Murthy et al., 2011). However, in vivo or animal cell culture-based methods are now being used to confirm the antimitotic, cytotoxic, or anticancerous activity of these compounds (Sreejaya et al., 2013). A handful of alternative animal-based in vivo methods (other than mice) have been reported using sea urchin embryos, brine shrimp, or embryos of zebra fish (Ved et al., 2010; Semenova et al., 2011; Li et al., 2012). These methods may be useful in terms of high-throughput analysis and low expense compared to methods that require an animal cell culture facility.

Here we introduce the earthworm, Eudrilus eugeniae, as an alternative inexpensive and novel in vivo system to identify compounds with antimitotic potential. This species possesses the ability to regenerate lost segments if they retain the clitellum region, a thick cylindrical collar-like structure present in the anterior part of the body (Samuel et al., 2012). Amputated earthworms with clitellum regions were used for the experiments, as the regeneration of tissues must go through rapid mitosis for blastema development. Amputated earthworms were tested with a commercially available antimitotic compound, colchicine, or with the extract from the rhizome of A. calamus L. (Acoraceae), which has been reported to have cytotoxic or antitumor properties (Bhattacharyya et al., 2008; Rajkumar et al., 2009; Imam et al., 2013).

It has been reported that the US National Cancer Institute has been performing prescreening through a 3-cell panel having SF-268 CNS, NCI-H460 lung, and MCF-7 breast cancer cells (Pan et al., 2010). The purpose of this study is to develop a simple and reliable animal-based in vivo system to prescreen hydrophilic antimitotic compounds, which could be used worldwide, even with limited facilities. This new system would help to accelerate the prescreening of new drug molecules. We have used

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commercially available colchicine and the aqueous extract of rhizomes of *A. calamus* to validate this new model system. To confirm the antimitotic activity, both in vitro and in vivo experiments were performed. We propose that earthworms could be used as a simple, less expensive, and novel system to prescreen antimitotic compounds.

### 2. Materials and methods

#### 2.1. Plant materials

The rhizomes of *A. calamus* L. and bulbs of *Allium cepa* L. were purchased from herbal stores or local markets and authenticated by Dr V Ganesan (taxonomist) in the Department of Botany at ANJAC College, Sivakasi, Tamil Nadu, India.

#### 2.2. Chemicals

Hematoxylin stain and antibodies were purchased from Sigma-Aldrich, India. All other analytical grade chemicals and solvents were purchased from HiMedia or Merck Ltd., India.

#### 2.3. Preparation of rhizome extract and colchicine

Rhizomes of *A. calamus* were washed thoroughly, air-dried, and powdered. Each 1 g of the powdered sample was extracted with 10 mL of sterile distilled water and kept overnight in a shaker at room temperature. Water-extracted samples were filtered with Whatman No. 1 filter paper, followed by a 0.2-µm filter. Water was evaporated with a water bath and the resultant residue was weighed and dissolved in sterile distilled water and stored in a freezer. When required, 100 mg/mL stock was prepared and diluted 100 times before use (1 mg/mL). Colchicine (Cat. No. RM342, HiMedia, India) was dissolved in sterile distilled water to prepare 10 mg/mL stock solution and diluted with water to prepare 100 µg/mL working solution.

#### 2.4. *Allium cepa* root tip assay

The *A. cepa* root tip assay was performed as described earlier (Kundu et al., 2016). Briefly, *Allium cepa* bulbs were kept in distilled water in 100-mL beakers under laboratory conditions to initiate root germination. Onion bulbs with newly germinated roots were incubated with distilled water, colchicine, or water extract of *A. calamus* rhizome for 16 h. Onion bulbs treated with distilled water were used as the control while colchicine served as the positive control. Following the incubation period, root tips were fixed with ethanol and acetic acid (3:1) for 10 min and washed with sterile distilled water. Root tips were treated with 1 N hydrochloric acid and kept for 10 min in a 60 °C water bath. Root tips were washed with sterile distilled water, stained with 0.5% safranin stain (W/V), and observed under a light microscope (40×). Chromosome morphology and stages were observed to calculate mitotic index. The mitotic index was calculated using the formula given below.

\[ \text{Mitotic index percentage (M.I)} = \frac{\text{Total number of cells in mitosis}}{\text{Total number of cells counted}} \times 100 \]

#### 2.5. Culture and maintenance of earthworm

Adult earthworms *E. eugeniae* were collected from the Agricultural Support Centre (Plant Clinic Centre), Tamil Nadu Agricultural University, Tirunelveli. *E. eugeniae* was maintained in a plastic tub containing soil, cow dung, and leaf litter in appropriate moisture conditions.

#### 2.6. Toxicity studies

For toxicity studies, colchicine was prepared as 10 µg/mL, 100 µg/mL, and 1 mg/mL using sterile distilled water from the stock of 10 mg/mL. For rhizome extract, 10 mg/mL or 1 mg/mL was prepared from the water bath’s evaporated residue as described in Section 2.3. These samples were tested on amputated earthworms (as described in Section 2.7) or onion roots, and their phenotype was monitored for 8 days.

#### 2.7. Earthworm regeneration assay

In this study, the earthworm regeneration assay is used to check the antimitotic potential of a known or unknown compound. Healthy adult earthworms of similar sizes, ranging from 1.0 to 1.5 g in body weight, were carefully selected for the experiments. Earthworms were cut with a sterile scalpel blade on the 30th segment from the mouth and the posterior segments were amputated, while the anterior parts of the body including the clitellum region were kept in separate beds and monitored every 24 h, with special attention paid to the amputation region for wound healing and/or formation and growth of the blastema.

Earthworms were injected between the clitellum and the amputation region with 10 µL of distilled water or aqueous drug solution. Injections were performed at 24-h intervals for 3 or 7 consecutive days. Five worms were used for each treatment and all experiments were performed at least 3 times.

#### 2.8. Stereo zoom microscope analysis

Amputated regions of earthworm were observed from the first day to the seventh day to record the morphological changes that occurred during blastema development. Worms were anesthetized with 10% ethanol before observation under a Nikon SMZ800 Stereo zoom microscope (Kalidas et al., 2015).

#### 2.9. Histology

The structural changes of the amputated regions of the earthworms at the cellular level were studied using histology as described earlier (Kalidas et al., 2015). After 3 days of treatment, earthworm tissues near the amputated regions were used for histological analysis. The earthworm tissues were fixed with 10% formaldehyde for 24 h and dehydrated using gradient isopropanol treatment (70%,
80%, 90%, and 100%, each for 1 h). Tissue clearing was subsequently achieved by treatment with xylene for 45 min, followed by wax impregnation. The 6-µm thin sections were made from the middle part of the block by microtome (Besto). The wax in the tissue sections was removed by washing with xylene and isopropanol. Later, tissues were stained with hematoxylin and eosin. The stained slides were mounted with DPX and documented under an Olympus BX53 microscope. Presence or absence of antimitotic activity was observed by noting healed wounds or development of the blastema respectively in the amputated regions.

2.10. Immunohistochemistry
To identify whether mitotic cell division was inhibited or not, earthworms injected with water, colchicine, or aqueous extract of rhizomes were subjected to immunohistochemistry (as described by Subramanian et al., 2017) with primary monoclonal antibody of anti-PCNA (Cat. No. WH0051111M2, Sigma-Aldrich, India) or anti-Phospho H3 [pSer10] (Cat. No. H0412, Sigma-Aldrich, India). Paraffin-embedded earthworm tissues were sectioned to 6 µm in thickness and dewaxed with xylene. Rehydrated sections were incubated for 30 min with freshly prepared 10% H₂O₂ and 10% methanol in phosphate buffered saline (1X PBS) to inhibit endogenous peroxidase activity. These sections were later treated with 0.1% trypsin in 0.1% CaCl₂ at 37 °C for 10 min. To avoid nonspecific binding or staining, sections were treated with 2% bovine serum albumin (BSA) in 1X PBS to inhibit endogenous peroxidase activity. These sections were later treated with PBS buffer and incubated with secondary antibody (goat antimouse IgG conjugated with horseradish peroxidase) at a dilution of 1:2000 in 1X PBST (Tween 20). Peroxidase activity was identified with a DAB Kit (GeNei), and the sections were counterstained with Ehrlich’s hematoxylin. The slides were mounted with DPX to document observations under an Olympus BX53 microscope. Brown spots resulting from agglutination reaction were counted for all slides prepared from each respective group of worms. Positive cells were counted from 10 different random images (40×) of each treatment and divided by the total number of cells.

2.11. Statistical analysis
Statistical analysis was performed (mean, standard deviation, and standard error) with Tukey’s multiple comparisons test in the software tool GraphPad Prism 6.0. P < 0.05 was considered statistically significant.

2.12. MTT assay
An assay with the MCF-7 cell line (human breast carcinoma) was performed at the JSS College of Pharmacy, Ooty, Tamil Nadu, as described earlier (Denizot and Lang, 1986). The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 × 10⁶ cells/mL using DMEM medium. To each well of a 96-well microtiter plate, 100 µL of the diluted cell suspension (approximately 10,000 cells/well) was added. After 24 h, the monolayer was washed once with medium and 100 µL of different test sample concentrations prepared in maintenance media were added per well. The plates were then incubated at 37 °C for 48 h in a 5% CO₂ atmosphere and microscopic examination was carried out. After 48 h, the sample solutions in the wells were discarded and 20 µL of MTT (2 mg/mL) in MEM-PR (MEM without phenol red) was added to each well. The absorbance was measured using a microplate reader at a wavelength of 540 nm.

The percentage of growth inhibition was calculated using the following formula and concentrations of test samples needed to inhibit cell growth by 50% values (IC₅₀) were generated from the dose–response curves for each cell line.

\[
\text{% of cytotoxicity} = \left( \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \right) \times 100
\]

3. Results

3.1. Toxicity studies of colchicine and aqueous extract of Acorus calamus rhizome
A concentration of colchicine of 1 mg/mL was lethal to earthworms and caused stunted or deformed growth in A. cepa root tips. We used 100 µg/mL as it did not affect the growth or phenotype of A. cepa roots or earthworms (Levan, 1938; Kundu et al., 2016). Aqueous extract of A. calamus rhizome (10 µL) was tested at two concentrations, 1 mg/mL or 10 mg/mL; neither was toxic to worms (data not shown). Toxicity was assessed by the presence or absence of deformation of worm tissue in any body part or autotomy. Since neither concentration affected the worm phenotype, 1 mg/mL was chosen for all remaining experiments.

3.2. Allium cepa root tip assay to test antimitotic activity
Preliminary cytotoxicity or antimitotic studies began with the A. cepa root tip assay, and further confirmation was done with in vivo or in vitro assays using mouse, human, or cancer cell lines. A. cepa root tip assay was performed to confirm that colchicine and the aqueous extract of rhizomes of A. calamus exhibited antimitotic properties. Root tips of A. cepa treated with water showed all 4 stages of mitotic cell division (Figure 1A). Root tips treated with colchicine showed more metaphases than other phases of mitosis (Figure 1B). Interestingly, root tips that were treated with the aqueous extract of rhizomes showed only a few metaphases or anaphases but showed more prophases (Figure 1C). Panels in Figure 1 (1D–1F) represent the type of mitotic phases observed in water,
colchicine, and aqueous extract of rhizomes, respectively. Colchicine- and rhizome extract-treated root tips showed fewer dividing cells. To get quantitative data, the mitotic index was calculated following all 3 treatments. Aqueous extract of A. calamus rhizomes and colchicine significantly decreased the mitotic index of A. cepa cells compared to control (water-treated) samples (Figure 1G). This strongly suggests that the aqueous extract of rhizomes has antimitotic compounds.

3.3. Validation of earthworm regeneration assay

The earthworm E. eugeniae is a segmented worm with a clitellum that divides the worm into anterior and posterior regions (Figure 2A). Regeneration of the blastema from amputated regions has been reported in this species (Samuel et al., 2012). Regeneration of the blastema from amputated regions, which requires rapid mitotic cell division, is generally observed 3 days after amputation. Regeneration ability of amputated regions was used to validate the antimitotic potential of the compounds. Samples of sterile distilled water (control), colchicine, or aqueous extract of A. calamus were injected once every 24 h for a period of 7 days. Wounds in the amputated regions of all worms were healed within 24 h. The regeneration of the blastema was observed after 3, 5, or 7 days in all control worms (Figures 2B–2D). The development of the blastema was inhibited by colchicine until 3, 5, or 7 days after amputation (Figures 2E–2G). This indicates that colchicine inhibits mitosis in the amputated region; hence, the blastema was not observed. Development of the blastema was observed in these worms when injection of colchicine was stopped (data not shown). Similarly, regeneration of blastema was not observed in worms that were injected with extract of A. calamus rhizomes 3, 5, or 7 days after amputation (Figures 2H–2J). This demonstrates that extracts of A. calamus possess compounds with antimitotic activity. Hence, this assay could be used to validate unknown antimitotic compounds.

3.4. Confirmation of antimitotic activity by histology and immunohistochemistry

Inhibition or development of the blastema in the amputated regions was verified by stereo zoom microscope. Panels of Figure 3 (3A–3C) show that blastemas were observed in water-treated samples (Figure 3A) and inhibited in colchicine- or rhizome extract-treated worms (Figures 3B and 3C). In order to study the inhibition of blastema formation further, blastemas (with adjacent segments) were processed for histology. Earthworms that were injected with water, aqueous extract of rhizome, or colchicine were processed for histological analysis after 3 days of treatment. Staining with hematoxylin and eosin and observation under a light microscope showed the presence of the outermost epithelial cell layer (ECL), the middle circular muscle layer (CML), and the innermost longitudinal cell layer (LCL) in the skin region. These 3 layers were very clear in control worms compared to the other worms (Figure 3D); specifically, the ECL and LCL layers are thicker in control worms and thinner in the treated worms. Among the 3 skin layers, the innermost LCL of the skin grew and extended as blastema only in the worms that were injected with sterile distilled water (Figure 3D). This innermost layer must undergo active mitotic cell division for blastema development (Santos et al., 2002). Earthworms that were injected with colchicine or aqueous extract of A. calamus did not show any further growth of the innermost layer development into blastema (Figures 3E and 3F). However, wound healing at the site of the amputation was observed. Histology studies confirmed that there was no development of blastema in the worms injected with either colchicine or rhizome extract, which suggests that the aqueous extract of rhizome possesses antimitotic activity similar to that of colchicine.

To demonstrate inhibition of cell division, tissues in the amputated region were taken for immunohistochemical analysis 3 days after the injection of all 3 samples. PCNA is a cell cycle marker protein, expressed only in S (synthesis)-phase cells and abundant in actively dividing cells (Foley et al., 1991). Phospho serine 10 histone H3 protein is expressed only in M (mitosis)-phase cells; hence, more of it is found in tissues where mitosis is inhibited (Subramanian et al., 2017). Antibodies of these 2 mitosis-specific proteins were used to find the mitotic cells in the blastema and nearby segments of the regenerating earthworms. Positive cells were calculated as described in Section 2. Figure 4A shows that the tissues of the control worms injected with sterile distilled water have more PCNA-expressing cells, which indicates active cell division. More PCNA is available in the synthesis phase of cells. The comparison of the status of PCNA-bound agglutinations in the groups of worms injected with either colchicine or extract of A. calamus clearly shows that agglutinations with PCNA were significantly reduced (Figures 4B and 4C). In contrast, expression of phospho serine 10 histone H3 was lower in control worms (Figure 4D). Interestingly, expression of this protein was enriched significantly in the worms injected with colchicine or extract of A. calamus (Figures 4E and 4F, respectively). Statistical analysis showed significant differences in expression of PCNA protein between the control worms and those treated with colchicine or the rhizome extract when counting PCNA antibody-bound positive cells against the total number of cells (Figure 4G). This suggests that the number of cells in the synthesis phase of mitosis is significantly greater in control worms compared to worms injected with colchicine or the rhizome extract. The data clearly confirm that the cell division potential has been reduced significantly by the injection of colchicine or extract of A. calamus. Statistical
Figure 1. Inhibition of cell division in *Allium cepa* root tips by colchicine and water extract of *Acorus calamus*. All root tips were incubated with respective samples for 16 h before the processing. A) Different stages of mitosis of roots treated with distilled water, observed with a 40× objective lens; B) the arrest of cell division predominantly in metaphase by colchicine (100 µg/mL); C) more prophase compared to the stages of metaphase and anaphase in samples treated with *Acorus calamus* (1 mg/mL); D) magnified images of all 4 phases of mitosis (prophase, metaphase, anaphase, and telophase) in water-treated samples; E) the magnified images of 6 metaphases and 1 anaphase in colchicine-treated samples; F) the magnified images of metaphase, anaphase, and prophase. The graph (G) shows the % of cells observed in different phases of mitotic cell division with mean ± SEM bar and P-value (***: P < 0.05). Marked circles indicate the different phases of cell division.

Data with phospho serine 10 histone H3 antibody shows that its expression was lower in control worms (Figure 4H) compared to that of worms treated with colchicine or the rhizome extract, which indicates that in control worms many cells in and around the amputated region passed the mitosis phase and moved to the next phase of the cell cycle. This suggests that many cells in worms treated with colchicine or the rhizome extract are in the mitotic phase, as the cell division is inhibited. It appears that colchicine arrested the cell division at the mitotic stage, possibly by inhibiting the polymerization of microtubules, as has been reported earlier (Jordan et al., 2004). Therefore, if colchicine arrests cell division, the number of cells that would go through the synthesis phase would be lower. This is clearly reflected by the number of PCNA antibody-bound positive cells in worms treated with colchicine. It is also in agreement with the number of phospho serine 10 histone H3 antibody-bound positive cells in the worms treated with colchicine; worms injected with colchicine have a high number of cells in the mitotic phase. Immunohistochemistry data suggest that colchicine inhibited the cell division in the amputated region better than the rhizome extract of *A. calamus*. The data clearly show that the extract of *A. calamus* has an antimitotic compound that can arrest the cell cycle at the mitotic stage. Although there was no blastema development due to the inhibition of cell division, the wounds of these worms were healed at the amputation sites within 24 h like those of the control worms. Our data suggest that earthworm regeneration in amputated regions could be exploited to validate antimitotic compounds.

3.5. Validation by MTT assay
To test the authenticity of the earthworm regeneration assay, MTT assay using the MCF-7 breast carcinoma cell
Figure 2. Inhibition of blastema development in *Eudrilus eugeniae* by colchicine and aqueous extract of *Acorus calamus*. A) The adult earthworm, *E. eugeniae*, is marked to show anterior (ar), clitellum (cl), and posterior (pr) regions. The control worms were injected with distilled water every 24 h for a period of 7 days, and the development of the blastema was observed after day 3 (B), day 5 (C), or day 7 (D). Colchicine was injected similarly for 7 consecutive days and the development of blastema was not observed after day 3 (E), day 5 (F), or day 7 (G). Aqueous extract of *A. calamus* rhizomes was injected every 24 h for a period of 7 days and the development of blastema was not observed after day 3 (H), day 5 (I), or day 7 (J). rb - regenerating blastema, wh - wound healing.
The MTT assay can be used to measure cytotoxicity or cytostatic activity. Colchicine inhibited cell proliferation better than the aqueous extract of rhizomes. However, both colchicine and rhizome extract inhibited cell proliferation and it was dependent on the concentration used. The half-maximal inhibition concentration (IC₅₀) values of colchicine and rhizome extract were 3.17 µg/mL and 76.11 µg/mL, respectively. Cells appeared healthy in the control treatment (Figure 5A). Inhibition of cell proliferation of MCF-7 breast cancer cells by colchicine (Figure 5B) and the rhizome extract (Figure 5C) was observed, as their cell morphology was different in size and shape compared to how cells appeared in the control.

4. Discussion
Compounds obtained from plants or other natural resources may provide drugs or leads for potential ingredients for cancer treatment (Khazir et al., 2014). Antimitotic compounds isolated from plants are currently being used as chemotherapeutic agents to control cancer disease. For example, microtubule-stabilizing compounds like taxol and its derivatives have been on the market to treat ovarian cancer, breast cancer, and non-small-cell lung cancer for more than 35 years. Chemotherapy is done with a mixture of compounds that inhibit cell division (Mukhtar et al., 2014). Development of resistance to antimitotic drugs is one of the issues of chemotherapy (Vuuren et al., 2015). Another problem is that compounds like taxanes and vinca alkaloids are hydrophobic in nature and are transported outside the cell by efflux pumps (Cella et al., 2003; Baldo et al., 2013). Therefore, to identify hydrophilic antimitotic compounds, the aqueous extract of *A. calamus* rhizomes was tested. All of our results suggest that the rhizome of this plant contains a hydrophilic antimitotic compound. However, more compounds should be screened worldwide to discover new active ingredients to treat cancer. Cancer cell lines or in vivo animal models in

Figure 3. Microscopy analysis of blastema development. Worms treated with water showed development of blastema 3 days after amputation (A), whereas wound healing alone was observed in worms treated with colchicine (B) or aqueous extract of *A. calamus* (C). Healed wounds are indicated by arrows. Histological studies show the regenerating budding tissue in water-treated worms (D), healed wounds alone in colchicine-treated worms (E), or aqueous extract of *A. calamus* (F). All tissues (D, E, and F) were stained with hematoxylin and eosin and were photographed with 4× magnification using a light microscope. The arrows in panels D, E, and F indicate the borders of the lesion. rb - regenerating blastema, wh - wound healing, ECL - epithelial cell layer, CML - circular muscle layer, LCL - longitudinal cell layer. Scale bar equals 50 µm.
use to examine cytotoxic, antimitotic, or anticancerous properties are expensive (Tannous et al., 2013). We need a simple system to screen more compounds anywhere in the world, even with limited facilities. We have demonstrated such a system here with earthworms to prescreen unknown compounds for their antimitotic potential. This system was validated with the commercially available known antimitotic compound colchicine and aqueous extracts of rhizomes of *A. calamus*.

Our results with the onion root tip assay agree with those of a previous report where the mitotic index was reduced with the aqueous extract of the *A. calamus* rhizome (Rajkumar et al., 2009). However, the authors referred to it as cytotoxic rather than antimitotic. Cytotoxicity-specific features, such as a micronucleus or chromosomal aberrations in root tip cells, were not observed with the colchicine or rhizome extract at the concentrations we used. Colchicine has been shown to have cytotoxicity and antimitotic potential at concentrations of 0.4 mg/mL and 0.1 mg/mL, respectively (Kundu et al., 2016). Hence, the same compound at higher concentrations might be

<table>
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<th>Sample</th>
<th>IC$_{50}$ (µg/mL)</th>
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<tr>
<td>Colchicine</td>
<td>3.17</td>
</tr>
<tr>
<td><em>A. calamus</em> extract</td>
<td>76.11</td>
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**Figure 4.** Immunohistochemistry study with mitosis-specific antibodies: Panels A, B, and C show the analysis of worms treated with water, colchicine, or aqueous extract of rhizomes, respectively, and tested with PCNA antibody. A) High number of PCNA positive cells is shown with 40× lens. Lower number of PCNA-positive cells is shown in panels B and C. Panels D, E, and F show the analysis of worms treated with water, colchicine, or aqueous extract of rhizomes respectively, treated with phospho serine 10 histone H3 antibody. D) A low number of histone H3-positive cells is shown with 40× magnification. A high number of H3-positive cells is shown in panels E and F. Quantitative analysis of positive cells with PCNA (G) and histone H3 (H) cells are shown with mean ± SEM bar and P value (**: P < 0.05). Scale bar equals 50 μm.
The concentration of colchicine used in our experiments was 0.1 mg/mL. It has not yet been reported that the aqueous extract of *A. calamus* has antimitotic properties. Our results suggest that aqueous extract of *A. calamus* rhizome inhibits mitosis in onion root tips, as the mitotic index was reduced and chromosomal abnormalities were not found. The onion root tip assay has been used worldwide for many years to prescreen antimitotic compounds and to study toxicity as well. Likewise, earthworms can be used as a simple in vivo animal-based system to prescreen antimitotic compounds. It is expected that active mitotic cell division in amputated regions of the earthworm is required for blastema development after wounds heal. Hence, the regeneration potential of amputated earthworms can be exploited to examine the potential of antimitotic compounds. Earthworms can also be used to study the genes involved in the regeneration of tissues. The role of the TCTP protein in the regeneration of tissues has been shown recently with amputated earthworms and cell division-specific antibodies (Subramanian et al., 2017).

In our experiments, all amputated earthworm wounds were healed within 24 h. Regeneration of the blastema was visible 3 days after amputation in control worms. However, regeneration of the blastema was not observed, even after 7 days, in worms injected with colchicine and aqueous extract of rhizomes. This finding was confirmed by stereo zoom light microscope. Histochemical studies of earthworms with amputations showed that the innermost LCL of skin layers extended to the blastema only in control worms. None of the skin layer was extended further for blastema development in worms treated with colchicine or aqueous extract of rhizomes, possibly due to their antimitotic properties. To confirm that the blastema development was affected by inhibition of cell division, an immunohistochemistry analysis with cell division-specific antibodies was performed. Antibodies of PCNA, found more in the synthesis phase of dividing cells, showed that worms injected with colchicine and aqueous extract of rhizomes have less agglutination compared to control worms. In contrast, antibodies of phospho serine 10 histone H3, found more in the mitotic phase of cells, showed that worms injected with colchicine and aqueous extract of rhizomes have more agglutination compared to control worms. This result indicates that worms injected with colchicine or rhizome extract did not have more cells with G1 phase or G2 phase when compared with control worms. This was further validated by MTT assay with the MCF-7 breast cancer cell line. Hence, the MTT assay can also be used to examine cytostatic cells; it would help to identify whether colchicine or compounds in the rhizome extract influence cell proliferation. Both colchicine and rhizome extract affected cell proliferation of breast cancer cells. There is a difference between the IC₅₀ values of colchicine and rhizome extract, which shows the difference between testing the purified antimitotic compound and the crude extract. Data from the MTT assay corresponded with earthworm regeneration assay and immunohistochemical assay results. Methanolic extract of powdered *A. calamus* plant has been shown to have an IC₅₀ value of 52.07 µg/mL (Sreejaya and Santhy, 2013). Methanolic extract of rhizome powder showed better IC₅₀ values compared to aqueous extract in an XTT assay using MDA-MB-435 and Hep3B cell lines (Rajkumar et al., 2009). However, our IC₅₀ values were higher with the breast cancer cell line (73 µg/mL), as the sample had been extracted with water rather than methanol. Methanol, an organic solvent, may extract the compound β-asarone from *A. calamus* rhizomes. It has been reported to affect cell division in cancer cell lines (Wu et al., 2015). β-Asarone is a volatile, lipophilic oil extracted by organic solvents (Shailajan et al., 2015). This suggests that our aqueous extract of rhizome

![Figure 5. Inhibition of cell proliferation by colchicine and rhizome extract: MTT assay with the MCF-7 breast cancer cell line was performed after 48 h of incubation of samples. The cells were cultured with various concentrations of colchicine and rhizome extract. Panels A, B, and C show the cells treated with sterile distilled water, colchicine, and aqueous extract of rhizomes, respectively. Concentrations at which colchicine and rhizome extract induced 50% of growth inhibition (IC₅₀) are shown here.](image-url)
possesses antimitotic compounds other than β-asarone. It could be one of the reasons for minor variations in the IC₅₀ values when compared to previously reported data. Earthworms are sensitive to organic solvents, so it is more suitable to examine water-soluble compounds. This would help to identify more hydrophilic compounds rather than hydrophobic compounds.

Hence, as the results from the aqueous extract of A. calamus with A. cepa root tip assay, immunohistochemical studies, and MTT assay correspond well with data obtained from colchicine, we conclude that the rhizome possesses an antimitotic compound. Our results indicate that the earthworm regeneration assay could be used as an alternative, novel, economical system for screening compounds with antimitotic potential. Experiments with cancer cell lines or the mouse in vivo model system require expensive facility infrastructures (Ryan, 1994), which may not be affordable for all laboratories around the world. Immunohistochemistry and MTT assays are expensive; we performed them only to validate the earthworm-based system. Selected samples after prescreening with earthworms may require further confirmation with in vitro or in vivo methods. In addition, obtaining an ethical clearance certificate may not be necessary for using earthworms in experiments, or may be relatively simple (Festing et al., 2007; Doke and Dhawale, 2015). This novel system can be adopted very easily by any researcher wherever earthworms can be grown.

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References


