Three Antimitotic Compounds Found in Banana Pseudo-Stem By Using Earthworms.

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Abstract

To identify antimitotic compounds from abundant and inexpensive plant resources, banana pseudo-stem was (BPS) chosen. Onion root tip assay and earthworm regeneration assay were carried out to test the antimitotic potential of aqueous extract of BPS. Earthworm (*Eudrilus eugeniae*) regeneration assay exploits the regeneration ability of amputated earthworms that retain the clitellum region. Aqueous extract of BPS decreased the mitotic index in *Allium cepa* root tips. Besides, this aqueous extract of BPS inhibited the regeneration of blastema from amputated earthworms as well. Validation of this extract with MTT (3-(4,5-dimethyl thiazolyl-2-yl)- 2,5-diphenyltetrazolium bromide) assay using MCF-7 breast cancer cell line further supported the presence of antimitotic compounds. Aqueous BPS extract was further fractionated with ethyl acetate and it was found to inhibit the regeneration of new tissues from amputated earthworms. Liquid Chromatography and Mass spectrometry (LC-MS) analysis was performed with aqueous BPS extract to predict the lead compounds. Prediction analysis with mass values revealed the presence of three different compounds viz. α -tocotrienol, 1,2,4-nonadecanetriol and 3',4',7-trihydroxyisoflavone, which were already reported to inhibit the cell division. All our results strongly supported that banana pseudo-stem extract possesses antimitotic compounds. This is the first report of identification of putative antimitotic compounds from aqueous extract of *Musa paradisiaca* var. Robusta by using earthworms.

Introduction

New beneficial aspects of earthworms are emerging in recent years, apart from vermicompost. This segmented invertebrate species is useful in assessing radiation hazards or evaluating the toxicity of chemical substances (Yesudhasan et al. 2013, 2018). Studies on the relationship between the central nervous system and the social ability of earthworm suggested its application towards research on social interaction (Gopi Daisy et al. 2016). Coelomic fluid of earthworms was demonstrated to inhibit the growth of fungal hyphae *in vitro* (Plavšín et al. 2017; Rajesh et al. 2019). Besides, coelomic fluid of earthworm species *Eudrilus eugeniae* has been shown to promote seed germination and the growth of legume seedling (Nadana Raja Vadivu et al. 2019). Moreover, coelomic fluid-treated rice plants were shown to have better disease resistance against the fungal pathogen in a rice plant (Nadana et al. 2020).

Antimitotic compounds play a crucial role in understanding of cell division at molecular level as well as in the development of new drug molecules to inhibit uncontrolled cell division. Hence natural products are one of the major resources; numerous extracts have been examined by MTT or other assays so far. Simple and inexpensive methods to identify such compounds would expedite the process of finding new drug leads. Earthworms were shown as a simple system to validate antimitotic compounds (Rajamanikkam et al. 2019). Banana pseudo-stems are left behind in the field after harvesting the fruits, or burnt in many fields although it can be used as a resource for production of bioethanol (Sadh et al. 2018). Hence, we examined the pith of banana pseudo-stem (BPS) of *Musa paradisiaca* to ascertain the compounds with antimitotic potential. Methanolic extract of the banana stem has been shown to induce wound healing in Wistar albino rats (Amutha et al. 2014). Ethanolic extract from banana's corm tissue

has been demonstrated to inhibit the calcium oxalate crystallization in kidneys and having diuretic effect (Kalpana et al. 2013). We have employed the regenerating ability of earthworm species *Eudrilus eugeniae* to validate the antimitotic potential of crude aqueous or differentially fractionated extracts of BPS. Our results strongly suggest that BPS extracts possess antimitotic compounds. Three different putative antimitotic compounds are predicted for the first time from the BPS by mass spectrometry analysis and their reported roles in arresting the cell division are discussed.

Materials And Methods

2.1 Plants and Chemicals:

BPS of *Musa paradisiaca* var Robusta was purchased from crop fields nearby the National research centre for banana (Indian council of agricultural research), Tiruchirappalli, Tamil Nadu, India. Onion (*Allium cepa* L.) bulbs were purchased from local markets and validated as described previously (Rajamanikkam et al. 2019). Purified colchicine, known antimitotic compound, other chemicals and solvents were purchased from Hi-Media and Merck Ltd. India.

2.2 Preparation of BPS pith extract:

The pith of pseudo-stem was washed completely with sterile distilled water followed by chopped into small pieces, shadow-dried and powdered. For every 1 gram of powdered sample, 10 ml sterile water was used and kept in a shaker for 12 h at 30° C to extract the compounds. This crude aqueous extract was filtered through the Whatman number 1 filter paper and subsequently with a 0.2-µm filter to remove microbes. Water bath set at 100°C was employed to evaporate the water completely from the aqueous extract. The dried residue was stored in the refrigerator using an airtight container. This was differentially fractionated further with four different solvents viz. chloroform, benzene, ethyl acetate, and ethanol. Ten ml of organic solvent was used for extraction from every 1 g of crude residue. Organic solvents in individual fractions were subsequently evaporated before dissolving again in sterile distilled water and were used for earthworm regeneration assays.

2.3 *Allium cepa* root tip assay:

This assay was performed as described earlier in Rajamanikkam et al. (2019). Briefly, newly sprouted onion roots with 3-5 cm were incubated for 16 h in distilled water, colchicine, or the aqueous extract of BPS. Later it was fixed and stained with safranin stain and viewed in the light microscope. The mitotic index was calculated as mentioned earlier (Rajesh et al. 2021).

2.4 Earthworm regeneration and MTT assay:

Earthworm regeneration assay has already been depicted before and followed similarly (Rajamanikkam et al. 2019). Briefly, mature adult healthy earthworms possessing clear clitellum were amputated with a sterile blade on the 30th segment from the mouth and kept in separate beds, and monitored every 24 h for the growth of blastema. Aqueous extracts or other samples (10 µl) to be tested were injected after the

wounds were healed in 24 h. Injections were carried out between the clitellum and the healed wounds at 24 h time intervals for 3 or 8 successive days. All experiments were carried out a minimum of 3 times having five similar healthy worms for each treatment. MTT assay with the MCF-7 cell line (Human breast cancer line) was performed at the JSS college of pharmacy, Ooty, Tamil Nadu, India as reported earlier. Growth inhibition percentage and IC50 value were estimated similarly as indicated before (Rajamanikkam et al. 2019).

2.5 Column chromatography:

The residue of aqueous extract was subjected to silica gel (60-120 mesh) column chromatography having dimensions of 30.5 × 2.5 cm. Chloroform was used to pack the column initially. Later, 0.5 g of aqueous extract residue was loaded to the column and extracted with chloroform: methanol: acetic acid (1:1:0.1). Collected flow-through was verified in thin layer chromatography to visualize the bands. Later, the organic solvent was evaporated by using water bath and the resultant residue was suspended in sterile distilled water and analysed in LC-MS.

2.6 LC-MS analysis:

The sample was processed at the Indian Institute of Science, Bengaluru Karnataka, India by employing the equipment as specified earlier (Nadana et al. 2020). Compounds were predicted based on the available mass values on web-based tools 'Competitive fragmentation modelling for metabolite identification', (<http://cfmid.wishartlab.com/identify>) and 'MassBank Europe' a high-quality mass spectral database (<https://massbank.eu/MassBank/Search>).

Results

3.1 Toxicity studies of aqueous extract of BPS

The toxicity level of colchicine was examined and finalized with 100 µg/ml as it did not affect the morphology of onion roots or earthworms as described earlier (Rajamanikkam et al. 2019). Aqueous extract of BPS (10 µl) was examined at 0.5 mg/ml, 1 mg/ml or 1.5 mg/ml concentrations; found out that none was toxic to earthworms (data not shown). Toxicity was assessed as described earlier (Rajamanikkam et al. 2019). Crude aqueous BPS extract at 1 mg/ml was adopted to use in all experiments unless specified otherwise.

3.2 BPS extract reduced mitotic index in onion root tips

Aqueous extract of BPS was examined for its antimitotic potential by using onion root tip assay. Commercially available colchicine, a known antimitotic compound, was used as a positive control. Onion root tips treated with sterile distilled water showed all 4 different phases of mitotic cell divisions viz. prophase, metaphase, anaphase, and telophase (Fig. 1A). Colchicine treated root tips showed many metaphases as it is known to inhibit the elongation of microtubule polymer (Fig. 1B). Interestingly, the aqueous extract of BPS-treated root tips exhibited many prophases compared to other phases of mitosis

(Fig. 1C). Cells showing the different mitotic phases in control (Fig. 1D), colchicine (Fig. 1E) or aqueous extract of BPS treated samples (Fig. 1F) are highlighted. Mitotic index was calculated to ascertain quantitative data of antimitotic activity of all three samples. Mitotic index was higher in control samples as there was no inhibition of cell division in the root tips. However, it was significantly lower in both colchicine and aqueous extract of pseudo-stem treated root tips (Fig. 1G). Colchicine inhibited the mitotic cell division better than the aqueous extract of BPS as illustrated. Both qualitative and quantitative data suggested that aqueous extract of BPS have compound(s) with antimitotic activity.

3.3 BPS extract inhibited blastema development in earthworms

Amputated adult earthworms having clitellum were used to validate the antimitotic potential of aqueous extract of BPS by exploiting the regeneration ability of earthworms. Development of blastema was monitored for 7 days in amputated earthworms that were injected with sterile distilled water, colchicine or the aqueous extract of BPS. Wounded cells in the amputated regions were healed within 24 h in all the earthworms. Earthworms that were injected with sterile distilled water exhibited the development of blastema on 3rd day and grown further on 5th and 7th day as shown (Fig. 2A, B and C). Colchicine injected earthworms did not develop any blastema from the healed region on 3rd, 5th or until 7th day as illustrated (Fig. 2D, E and F). Interestingly, the aqueous extract of BPS also inhibited the development of blastema from the healed region on 3rd, 5th or until the 7th day as demonstrated (Fig. 2G, H and I). Our results with earthworm regeneration assay strongly suggested that aqueous extract of BPS had compounds that interfere mitotic cell division.

3.4 Validation with MTT assay

MTT assay with the human breast carcinoma cell line (MCF-7) was performed to further validate the aqueous extract of BPS. MTT assay could be used for measuring cytostatic activity although used extensively for cytotoxicity. Various concentrations of BPS aqueous extracts such as 31.25, 62.5, 125 and 250 µg/ml were examined to ascertain its half-maximal inhibition concentration (IC₅₀ value). Cells treated with sterile distilled water were growing and dividing normally (Fig. 3A) whereas the BPS aqueous extract-treated cells were lower in number comparatively and had a difference in the size and shape (Fig. 3B). IC₅₀ value of aqueous extract was determined as 56.37 µg/ml (Fig. 3C). Purified colchicinewas used as a positive control,which exhibited the IC₅₀ value as 3.2 µg/ml (data not shown). These results further suggested that BPS had compound(s) that could interfere with mitotic cell division.

3.5 Fractionation of aqueous extract and their antimitotic potential

The compounds present in the aqueous extract of BPS were further fractionated by using their difference insolubility in various organic solvents. Hence, it would narrow down to fewer number of compounds compared to total crude extract. Aqueous extract of the BPS was fractionated separately with four different organic solvents such as benzene, chloroform, ethyl acetate or ethanol. Subsequent to differential fractionation, organic solvents were evaporated and the residues were suspended in sterile distilled water and subjected to earthworm regeneration assay. Sterile distilled water injected earthworms

made the development of blastema on 5th or 6th day and grown very well by 7th day (Fig. 4A). Colchicine injected earthworms did not show any development of blastema until by 7th day (Fig. 4B). Initiation and growth of blastema were observed in earthworms injected with fractions partitioned from benzene, and chloroform (Fig. 4C, 4D). However, blastema development was not detected from earthworms injected with fraction partitioned from ethyl acetate (Fig. 4E). Fraction portioned with ethanol did not inhibit the development of blastema completely as shown (Fig. 4F). Our results suggested that fraction partitioned by ethyl acetate might possess antimitotic compounds. Moreover, the putative antimitotic compound might have better solubility in ethyl acetate compared to other organic solvents such as benzene, chloroform and ethanol.

3.6 LC-MS analysis of aqueous BPS extract

The aqueous extract of BPS was processed through silica gel column as described in the methods section and the eluted fraction was subjected to LC-MS analysis to identify putative antimitotic compounds. Eight major compounds were identified from the total ion chromatogram, although 435 compounds were detected (Fig. 5). The best matching compounds, based on the mass values were predicted and listed in Table 1. The biological role or related information of these compounds is also listed in the table. Interestingly, three different compounds such as α -tocotrienol, 1,2,4-nonadecanetriol and 3',4',7-trihydroxyisoflavone found in the analysis were already reported to inhibit the cell division.

Table 1
Predicted metabolites by mass values and their reported biological function

S. No	Compound number	Mass value	Best match	Reported activity	Reference
1*	105	453.3444	α -Tocotrienoxyl radical	Antineoplastic	(Aggarwal et al., 2019)
2	108	475.3250	Trandolapril-d5 Diketopiperazine	ACE inhibitor used to treat high blood pressure	(Guay, 2003) (Borthwick and Liddle, 2011)
3*	131	701.4941	1,2,4-Nonadecanetriol	Cytotoxic agent	(Oberlies et al., 1998)
4	132	340.2608	15H-11,12-EETA	Regulation of the cerebral circulation	HMDB0005050 (Human Metabolome Database ID)
5	205	311.1842	Tovophyllin B	Anti-inflammatory	(El-Agamy et al., 2020)
6	207	344.3140	AFN911	Metabolite of Imatinib-tyrosine kinase inhibitor	HMDB0013863 (Human Metabolome Database ID)
7	276	455.4564	Bishomohopanoic acid	Not Known	PubChem-CID-21145182
8*	418	270.2797	3',4',7-Trihydroxyisoflavone	Inhibition of cell division	(Lee et al., 2013)
Star (*) indicates the compounds reported to affect the cell division.					

Discussion

Plants have been one of the main sources of lead molecules in the development of pharmaceutical products. Many antimitotic compounds including colchicine, vinblastine, vincristine and paclitaxel were developed from plants. The National Cancer Institute program in the United States is reportedly using 60 different cell lines in the primary screen to investigate anticancer compounds (Boyd and Paull 1995). We demonstrated recently that using simple inexpensive earthworm based *in vivo* system to pre-screen antimitotic compounds (Rajamanikkam et al. 2019). To validate this system further, BPS pith was chosen. This research would help to identify whether this low-cost banana stem could be used as a better bioresource material to extract pharmaceutically significant compounds.

Different parts of banana plant had already been explored to ascertain its therapeutic properties. Hexane and ethanol extract of banana fruit pulp and peels of *M. sapientum* exhibited strong cytotoxic activity in cancer cell line HCT 116 (Dahham et al. 2015). Besides, methanolic extract of banana inflorescence was

shown to inhibit the colon cancer cells by arresting the mitotic cell division at the G2/M phase (Arun et al. 2018). Ethyl acetate extract of banana roots (*M. paradisiaca*) was shown to induce apoptosis without arresting the cell cycle in Ehrlich ascites tumour cells (Raj M. et al. 2017). Interestingly, the exudate of BPS pith has been shown to possess antioxidant, antitumor and antibacterial properties. Biochemical tests to ascertain the phytochemicals of exudates revealed the presence of alkaloids, flavonoids, anthraquinone, cardiac glycoside and phlorotannin's (Abdel Ghany et al. 2019). Mass spectrometry analysis of banana stem extract with methanol (80%) showed the presence of many antidiabetic compounds (Dong et al. 2017). Although different species of the banana stem was used for extraction with different solvents and examined for their cytotoxic activities; compounds responsible for cytotoxicity or antimitotic activity have not been identified or proposed yet.

Analysis of aqueous extract of BPS with onion root tip assay did not show any cytotoxicity specific features such as chromosomal aberration or micronucleus in our experiments. Colchicine had been shown as cytotoxic at higher concentration (0.4 mg/ml) but antimitotic at 0.1 mg/ml (Kundu et al. 2017). Hence the colchicine was used at 0.1 mg/ml concentration in our experiments, cytotoxicity specific features were not observed in the chromosomes of onion roots. Earthworms' morphology was quite similar in both control (water injected) and in aqueous extract injected worms, during the course of injection for a period of 7 days, suggested that there was not any cytotoxicity in the BPS extract. Not observing the self-defence feature in the earthworm, autotomy, further supported the absence of cytotoxicity (Yesudhasan et al. 2018). The aqueous extract was further examined with MTT assay as it could be used to analyse both cytostatic and cytotoxicity of compounds. This assay with breast cancer cell line (MCF-7) demonstrated that the aqueous extract inhibited the cell proliferation (Fig. 3). The morphology of the cells was changed by aqueous extract and was very similar to cell morphology induced by the antimitotic compound, colchicines (Rajamanikkam et al. 2019). The IC₅₀ value of the aqueous extract of the banana stem in MCF-7 cell lines was matching with already reported value by ethyl acetate extract of banana stem (Raj M. et al. 2017). All these results strongly supported that the aqueous extract of the BPS has antimitotic compound(s).

Differential fractionation of the aqueous extract with benzene, chloroform, ethyl acetate or ethanol and subsequent earthworm regeneration assay demonstrated that ethyl acetate extraction might have the compounds to inhibit blastema formation. Although ethanol is highly polar than ethyl acetate, fractions partitioned with ethanol was not able to inhibit the blastema development completely. It suggested that the antimitotic compounds might be non-polar but became polar (water soluble) due to tight association with other compounds in the BPS. Flow-through of silica gel column and subsequent LC-MS analysis of BPS extract predicted 3 putative compounds that could arrest mitotic cell division (Table 1).

The α -tocotrienoxyl radical could be formed by the combination of α -tocotrienol and monodehydroascorbate (<https://metabolicatlas.org>) and it is the reversible reaction in the cytosol. This α -tocotrienol, vitamin E analogue, has been suggested to play significant roles in inhibiting cancer by arresting cell cycle checkpoints, apoptosis, anti-angiogenesis, anti-metastasis and antioxidant activities (Aggarwal et al. 2019). However, the precise mode of the mechanism is yet to be discovered. The second

predicted compound 1,2,4-nonadecanetriol has already been reported from unripe avocado fruits and shown to possess anticancer activity (Oberlies et al. 1998). This compound has already been listed in NPACT (Naturally occurring plant-based anticancerous compound-activity target) database with an ID of NPACT00024 (https://webs.iiitd.edu.in/raghava/npact/brws_alp.php?b=1). The third predicted compound 3',4',7-trihydroxyisoflavone is a 7-hydroxyisoflavone that is daidzein substituted by OH group at position 3' and has been reported as an antineoplastic agent. Metabolite of daidzein, 6,7,4'-trihydroxyisoflavone has been shown to inhibit the cell proliferation by arresting cell cycle at S and G2/M phases in human breast cancer cells (Lee et al. 2013).

Conclusions

We conclude that BPS have at least three compounds that could inhibit cell proliferation or arresting the mitotic cell division. However, purification of these antimitotic compounds and further studies are required to examine their potential in becoming pharmaceutically important compounds. BPS could be used as low-cost bioresource material to develop commercially valuable drug molecules in the future instead of wasting them. We have demonstrated that earthworms could be utilized as simple *in vivo* animal model system to perform screening of antimitotic compounds from BPS.

Declarations

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: Data sharing is not applicable to this article as no datasets were generated or analysed during the current study.

Competing Interest: The authors declare that they have no competing interests.

Funding: Grant money was not received to conduct this study.

Authors' contributions: CR performed earthworm regeneration assay and prepared samples for assay with MCF-7 cells. SKB performed onion root tip assay and performed extraction of samples from banana pseudo-stem. RP helped in designing column chromatography. NS designed the experiments with fractionation of aqueous extract with organic solvents. KP designed the experiments, supervised, interpreted and prepared the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1

Inhibition of cell division in onion root tips by aqueous extract of BPS. Root tips treated with distilled water (A), colchicine (B) or aqueous extract of BPS (C) were visualized in light microscope with 40× objective lens. Enlarged images of water treated samples with all 4 phases of mitosis (D); colchicine treated samples with metaphases (E) and aqueous extract-treated cells with a smaller number of

different phases (F) are shown. Scale bar indicates the length of 100 μm . The histogram (G) indicates the percentage of mitotic index (Y axis) with standard error mean (SEM) bar and P-value (***: $P < 0.05$).

Figure 2

Aqueous extract of BPS inhibits blastema development in *E. eugeniae*. The development of blastema from amputated earthworms that were injected with distilled water after 3 days (A), 5 days (B) and 7 days (C) are shown. Blastema development was not observed in earthworms that were injected with colchicine after 3 days (D), 5 days (E) and 7 days (F). Injection of aqueous extract of BPS inhibited the formation of blastema in worms on 3rd (G), 5th (H) or 7th day (I).

Figure 3

Aqueous extract of BPS interfere cell division in MCF-7 cancer cell line. MCF-7 breast cancer cells treated with sterile distilled water having a greater number of the normal shape of cells (A) whereas cells treated with aqueous extract of BPS having a fewer number of cells with different shapes (B) are shown. (C) Different concentrations of aqueous extract of BPS ($\mu\text{g/ml}$) are given in the X-axis and the % of the viability of cells is given in Y-axis. IC₅₀ value of BPS extract ($56.37 \mu\text{g/ml}$) was inferred.

Figure 4

Analysis of blastema development with differentially extracted BPS extract. (A) Blastema development in water injected earthworms after 5, 6 or 7 days of amputation; or (B) no such regeneration in earthworms injected with colchicine are illustrated. Regeneration of tissues in amputated earthworms injected with benzene (C) or chloroform (D) fractionated BPS extract is shown. (E) No such regeneration of tissues

from amputated earthworms was observed in ethyl acetate fractionated BPS extract. (F) Blastema development was observed in ethanol fractionated BPS extract.

Figure 5

Base peak chromatogram of BPS extract. Total ion chromatogram obtained after liquid chromatography, indicating the 8 intense peaks. The numbers marked along with the peaks indicate the number of the compound among the total number of compounds identified in the extract. The X-axis indicates the retention time and the Y-axis represents the signal intensity.

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