Trop J Nat Prod Res, June 2021; 5(6):1152-1157

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Euphorbia graminea Jacq. (Euphorbiaceae): *In-Vitro* Cytotoxic, Phytotoxic and Anti-Proliferative Assessment of the Extract and Fractions of the Leaves

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ARTICLE INFO

Article history:
Received 18 May 2021
Revised 20 June 2021
Accepted 27 June 2021
Published online 01 July 2021

ABSTRACT

The genus Euphorbia is known to have several medicinal applications in ethnomedicine. The objective of this work was to evaluate the biological properties of the extract and fractions of *E. graminea* using the Brine shrimp and Tadpole lethality, seed radicle and fronds inhibition as well as the anticancer activity using the SRB antiproliferative assays. The methanol extract of the leaves of *E. graminea* was partitioned using distilled water and chloroform. Phytochemical screening and the biological activities were carried out. The Brine Shrimp and Phytotoxic assays were carried out at concentrations between 10-1000 µg/mL, while the seed radicle, tadpole and SRB-antiproliferative assay were carried out at 1-30 mg/mL, 20-400 µg/mL and 1-250 µg/mL respectively for the extract and fractions of *E. graminea*. The chloroform fraction was observed to be 100% cytotoxic against the Brine Shrimps and Tadpoles at the highest concentrations of 1000 and 400 µg/mL, respectively. The crude extract and the chloroform fraction completely inhibited radicle growth at 30 mg/mL. For the phytotoxicity assay, an FI_{50} of 81.03 and 25.15 µg/mL was recorded for the chloroform fraction and paraquat, respectively. The chloroform fraction recorded LC_{50} of 72.54 µg/mL against 6.12 µg/mL by doxorubicin. These findings suggest the potential cytotoxic, phytotoxic and antiproliferative activities of the leaves of *E. graminea*.

Keywords: Cytotoxic, phytotoxic, Euphorbia, Brine Shrimp, fronds, *Sorghum*

Introduction

The genus Euphorbia is cosmopolitan, confined to tropical, subtropical, and warm temperate regions and consists of monoicous herbs, shrubs, or trees, often succulent, with milky latex and simple indumentums when present.[1] Leaves are frequently whorly or free, or connote, of three types, lower, median and upper, or ray. All and indeed most leaves are usually sessile, rarely briefly petiolated, simple, entire or toothed, and penni- or palm nerved.[2] In Nigeria, the Euphorbiaceae family includes about thirty species but *E. graminea* is not one of them.[3] This indicates that *E. graminea* is possibly an introduction to Nigeria recently. Moreover, most publications did not list previous records of its life.[4-6] The plant is an annual plant that grows to a height of 15–30 cm, sometimes taller, frequently branches from the base, and is dichotomously branched distally.[7] The leaves are alternating and are ovate or oblong with some wide distant teeth to the base of the plant, while they are elliptical to linear and complete. The seeds can be oval, white, black, or chalky-gray.[8] This plant, having been reported to be new to Nigeria has little or no reported ethnomedicinal uses. However, in Columbia, the country of origin, *E. graminea* has been reported to have some notable ethnomedicinal applications which includes the treatment of skin disorders, ulcers, cancers and tumors.[9]

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Citation: Ikpefan EO, Ayinde BA, Ahzah M. *Euphorbia graminea* Jacq. (Euphorbiaceae): *In-Vitro* Cytotoxic, Phytotoxic and Anti-proliferative Assessment of the Extract and Fractions of the Leaves. Trop J Nat Prod Res. 2021; 5(6):1152-1157. doi.org/10.26538/tjnpr/v5i6.29

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

At the moment, there are limited or scanty information regarding the biological activities of *E. graminea* . The work thus aims at assessing the cytotoxic, phytotoxic, and anti-proliferative potentials of the extract and fractions of the leaves of *E.graminea* using bench-top assays involving the use of Brine Shrimp Tadpoles, Seed radicle, Lemna weeds and SRB-antiproliferative assays respectively.

Materials and Methods

Collection of plant samples

The samples of the fresh leaves from the *E. graminea* were collected in February, 2019 at Capitol inside the University of Benin, Ugbowo campus, Edo State, Nigeria. An herbarium number (FHI 108339) was provided by Mr Adewale at the Forest Research Institute where the herbarium specimen was deposited. The leaves of *E. graminea* were spread on the laboratory table in an open air for 48 h following which they were dried in the oven at 45°C for 30 mins. The dried leaves were then ground in a laboratory milling machine, after which they were kept in air-tight containers. The powdered sample of the leaves (1.5 kg) was then extracted by maceration with 80% methanol (12 L) and the filtrate concentrated under reduced pressure to remove the solvent yielding the paste-like extract.

Fractionation of the extract

A total of 50 g of extract of *E. graminea* were dissolved in CH_3OH and distilled water in equal proportion (1:1 {200 mL}) and were exhaustively fractionated with chloroform (3× 200 mL) into Chloroform and aqueous fractions. Both fractions were separately collected, dried and stored in air sealed jars. The Extract and fractions of the leaves of *E. graminea* were investigated for the existence of phytochemicals using existing protocol.[10]

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Biological Assays
The extract and fractions of the leaves of *E. graminea* were subjected to some biological studies listed below.

Cytotoxicity on brine shrimp
The extract and fractions of *E. graminea* were screened for lethality using the Brine shrimp assay methods previously described. [11,12] Freshly hatched Nauplii were obtained from the Zoology Department of the University of Karachi, Pakistan and the experiment was carried out at the HEJ Research Laboratory, University of Karachi, Pakistan. The procedure involves the addition of 2 mL of distilled water to 20 mg of the extract. From the resulting stock solution, the various concentrations of 10, 100 and 1000 µg/mL were obtained by dilution in vials which were left to stand overnight following which 20 mL of seawater solution (38 g/L) was poured in each vial. After approximately 36 hours, ten adult nauplii larvae were placed into each vial and spread evenly across the surface with Pasteur pipette. The vials were held at low temperatures (25-27^{o}C) under illumination, and the positive controls comprised etoposide (10-100 µg/mL) augmented with brine solution in vials. The LC_{50} was calculated using the following regression equation; Y = ax + b. The experiment was repeated for the aqueous and chloroform fraction.

Cytotoxicity on tadpoles (Raniceps ranninus)
The extract and fractions of *E. graminea* were subjected to cytotoxicity assay involving the use of tadpoles of *Raniceps ranninus* using previously described protocols.[13,14] Freshly scooped hatched tadpoles of *Raniceps ranninus* were picked from a stagnated puddle of water near Abraka Beach Motel at Abraka, Delta State Nigeria. Ten tadpoles of uniform sizes were spotted and were picked with the aid of a Pasteured pipette into glass beakers containing 30 mL of the tadpole's natural water which was made up to 49.5 mL with distilled water and eventually to 50 mL with 0.5 mL of 20, 40, 100, 200 and 400 µg/mL of the extract of *E. graminea*. The experiment was observed for 24 h and was carried out in replicates of three and the procedure was repeated for the fractions.

Phytotoxicity on Lemna minor
The effects of the extract on fronds of *Lemna minor* were carried out at various concentrations as previously described.[15] The media was made by dissolving E-medium in distilled water (100 mL) and maintaining the pH between 6.0 and 7.0 by adding KOH solution. This was then followed by autoclaving of the media at 121^{o}C, for a period of 15 min. A portion of the extract (10 mg) was added to 1 mL of ethanol to make up the stock. The solvent was evaporated from the flask in the aseptic environment. In each tube, 20 mL of autoclaved medium was added alongside with ten plants, each with a rosette of three fronds. The experiment was repeated for the fractions as well as the control drug, paraquat (10-100 µg/mL). The growth inhibition percentage (%) was calculated with reference to the negative and positive (paraquat) controls. The treatment was replicated in triplicate for the water-soluble and chloroform fraction.

Growth inhibition on seed radicle (Sorghum bicolor)
The leaves of *E. graminea* extract and fractions were tested against fast growing radicle cells using the *Sorghum bicolor* inhibition assay previously reported. [16, 17] Prior to application, the guinea corn seeds obtained from a local market in at Abraka were disinfected with 70 % ethanol and air dried. Thereafter, 20 viable disinfected guinea corn seeds were later placed in petri dishes made of cotton wool laced with 90 mm filter paper. Following that, the various concentrations of 1, 5, 10, 20, and 30 mg/mL of the extract comprising 5% Dimethyl sulphoxide (DMSO) were added. The experiment was done in replicate of three.

Sulforhodamine-B antiproliferative assay
The SRB (colorimetric sulforhodamine B assay) method designed by Khatun *et al*, [18] and described by Skehan *et al*, [19] was used to assess cytotoxicity against mouse Hela cell lines provided by the cell bank of the ICCBS. The rapidly growing exponential cells were harvested and suspended in growth media in a 96-well plate (100 mL, RPMI1640). Following a 24-hour incubation at 37 ^{o}C in humidified 5 percent CO_2, serially-diluted test solutions of extract (1-250 µg/mL), fractions (1-100 µg/mL) and the control drug, doxorubicin (0.01-10 µg/mL) in 100 mL of RPMI medium were added to the wells and incubated for another 48 hours. The 50% Trichoride solution was used to fix the cells in in place and were examined using a phase-contrast microscope after stanning with an SRB solution. Unbound dye was loosened by four times washing with 1 percent acetic acid followed by addition of 10m Tris to extract the protein-bound dye for 5 minutes. The plates were read in a microplate reader at 520 nm (Tecan Sunrise microplate reader). In this case, the results are written in terms of Total Growth Inhibition (TGI), Lethal concentrations at 50% (LC_{50}), Growth inhibition at 50% (GI_{50}) all in microgram per milliliter (µg/mL).

Statistical Analysis
The data were analyzed with GraphPad Prism 7.0, and one-way analysis of variance (ANOVA) was employed, with $p < 0.05$ and $p > 0.05$ being regarded statistically significant and non-significant, respectively. The final results were displayed as Mean ±Standard Error of Mean.

Results and Discussion

A total of 76.65g extract was obtained from the powdered leaves sample (1.5kg) of *E.graminea* which is equivalent to 5.11 % of the powdered leaves sample. The fractionation of 50 g of the extract yielded 7.01 g (14.02%) and 21.68 g (43.36%) of the chloroform and aqueous fractions respectively. The phytochemical screening result of leaves of *E. graminea* showed variations in the distribution of the secondary metabolites among the extracts and fractions (Table 1). This could be attributed to the solubility of the metabolites in the various solvents used which could have influence in the biological activities recorded. The current work investigated the cytotoxic and phytotoxic potentials of the extract and fractions of *E. graminea* using simple bench-top assay methods involving the use of Brine shrimp and tadpoles lethality assay, antiproliferation against cancer cell line of Hela, as well as growth inhibitory assay against germinating seeds of *Sorghum bicolor* and fronds of *Lemna minor*.

Brine shrimp lethality test
The Shrimp larvae responded differently to different concentration levels of extract and fractions of *E. graminea* suggesting a dose-dependent activity. At test concentrations of 10, 100, and 1000 g/mL, the extract of *E.graminea* showed cytotoxicity of 16.67, 66.00, and 93.33 percent, with an LC_{50} of 68.80 µg/mL. While the aqueous fraction of *E. graminea* had cytotoxicity of 3.33 percent only at the maximum concentration, the chloroform fraction had cytotoxicities of 33.60, 76.60, and 100 percent at 10, 100, and 1000 g/mL with LC_{50} of 28.66 g/mL. However, this activity was considerably lower than etoposide (control drug), which produced 100% mortality at 1000 g/mL with an LC_{50} of 10.0 g/mL when used as a positive control (Table 2). Suraj and Chatterjee[20] has previously reported the higher cytotoxic activity of the chloroform fraction on brine shrimp, with an LC_{50} of 5 mg/mL compared to 15 and 20 mg/mL for the Benzene and Petroleum ether fractions of *Calotropis procera*, which is in line with our work.

Tadpole Mortality assay
There were variations in the sensitivities of the tadpoles to the constituents of the extract and fractions of *E. graminea*. The methanol extract of *E. graminea*, gave maximum mortalities of 100 % at 200 and 400 µg/mL at a time of 80.4 and 38 minutes respectively. At 20 and 200 µg/mL the chloroform fraction of *E. graminea* produced average mortalities that were statistically different from those of control. However, at 400 µg/mL, 100 % mortality was recorded for the chloroform fraction (Table 3). The work of Ayinde and Ewoigbokhan,[21] in their previous work has similarly shown the higher effects of the chloroform fraction over the aqueous fraction of *Ficus exasperate* with LC_{50} of 28.67 and 57.33 µg/mL which support our work.

ISSN 2616-0684 (Print)
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Phytotoxicity assay

The phytotoxicity results according to Khurm *et al*, [22] was classified as low (% inhibition 40%), moderate (% inhibition = 40-50%), and significant (50-100%) and was interpreted as FI_{50}. Using this classification, the extract and the aqueous fraction of *E. graminea* can be said to be inactive. However, at all concentrations, the activity of the chloroform fraction was observed to be highest with FI_{50} of 81.03 µg/mL which was significantly lower than that of the positive control (paraquat) whose FI_{50} was 25.15 µg/mL (Table 4). This results corroborate studies by Bashir *et al*,[23] which highlighted the phytotoxic potential of the chloroform fraction over the hexane, ethyl acetate, n-butanol, and aqueous fractions of *Myrsine africana* .

Seed radicle assay

The extract of *E. graminea* was observed to remarkably inhibit the growth of the guinea corn radicles and the inhibitory effects were sustained all through the period of the experiment (Figure 1). At 24 h, seeds treated with 5 and 10 mg/mL of the chloroform fraction gave average radicle lengths of 1.12 ± 0.09 and 0.43 ± 0.08 mm compare to 3.68 ± 0.63mm produced by the control seeds. There was complete growth inhibition of seeds treated with 20 and 30 mg/mL concentrations. At the end of the experiment, (after 96 hr), the control seeds had an average length of 58.68 ± 5.24 mm while seeds pre-treated with 1, 5 and 10 mg/mL of the aqueous and chloroform fractions showed an average lengths of 30.88 ± 3.57 mm (47.38 % inhibition), 5.90 ± 1.11 mm (89.95 % inhibition) and 2.83 ± 0.52 mm (95.2 % inhibition) respectively. Seed germination at 20 and 30 mg/mL were completely inhibited by the chloroform fraction. The variations in the growth inhibition were observed to be significant at $P < 0.05$ (Figure 2). Unlike the chloroform fraction, seed germinations were not completely inhibited when treated with 20 and 30mg/ml of the aqueous fraction as they produced 7.05 ± 1.05 and 3.42 ± 0.87 mm respectively compared to 41.89 ± 6.68 mm produced by the control after 96 hr incubation period (Figure 3).

Cancer cell line antiproliferative assay

The presence of potent cytotoxic bioactive compounds in the leaves of *E. graminea* as demonstrated by the brine shrimp, tadpole, phytotoxic and seed radicle assays was further correlated by the outcome of the chloroform fraction in the antiproliferative assay on the Hela cancer cell lines. The higher activity of the chloroform fraction was also observed in the tadpole cytotoxic and phytotoxicity studies. This goes to suggest that the active metabolites responsible for the observed activities resides more in the chloroform phase. According to Bhatti *et al*.,[24] the Brine Shrimp assay is a globally approved, less expensive process for evaluating antitumor properties. This became evident in the results of the effects of the extract and fractions of *E. graminea* on Hela cancer cell lines. The results of the SRB cytotoxic assay also suggest the potency of the chloroform fraction of *E. graminea* over the extract and aqueous fraction as it recorded GI_{50}, LC_{50} and TGI of 68.0, 72.54 and 54.57 µg/mL on the Hela cell lines against 0.17, 6.12 and 8.72 µg/mL recorded by the control drug (Doxorubicin). However, the extract as well as the aqueous fraction recorded GI_{50}, LC_{50} and TGI greater than 250 and 100 µg/mL respectively (Table 5). The works of Ahmad *et al*, [25] had previously demonstrated the higher activity of the chloroform fractions of *Rumex hastatus* and *Alstonia scholaris* on HeLa cell lines which is in line with our work.

Table 1: Result of the preliminary Phytochemical screening of the extract and fractions of *Euphorbia graminea*

Phytochemical class	Extract	Fractions	
		Aqueous	Chloroform
Alkaloid	+	+	-
Anthraquinones	+	+	+
Cardiac glycoside	+	+	+
Flavonoids	+	+	+
Saponins	+	+	-
Steroids	+	-	+
Tannins	+	+	+
Terpenes	+	-	+

Key: + = Present; - = absent

Table 2: Cytotoxic effects of the extract and fractions of *E. graminea* against Brine shrimps nauplii

Sample	Percentage Mortality (%) Concentration (µg/mL)			
	10	100	1000	LC_{50} (µg/mL)
Extract (EX)	16.67 ± 0.70	66.00 ± 3.97	93.33 ± 6.30	68.80
Aqueous fraction (AQF)	0.00 ± 0.00	0.00 ± 0.00	3.33 ± 0.30	>1000
Chloroform fraction (CHF)	33.64 ± 0.28	76.60 ± 1.83	100 ± 0.00	28.66
Ectoposide (+ve control)	50.30 ± 0.5	89.10 ± 1.98	100 ± 0.00	10.00
Distilled water (-ve control)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	-

Values are expressed as the mean ± SEM of three independent observations, n=10

Table 3: Cytotoxic effects of the extract and fractions of *E. graminea* on tadpoles

Sample	Percentage Mortality (%) Concentrations (ug/mL)					
	20	40	100	200	400	LC_{50} (ug/mL)
Extract	0.00 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	3.33 ± 0.01	> 400
Aqueous fraction	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	> 400
Chloroform fraction	6.67 ± 0.02	23.33 ± 0.08	53.33 ± 0.12	100 ± 0.00	100 ± 0.00	297.5
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	> 400

Values are expressed as the mean ± SEM of three independent observations, n=10

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Table 4: Phytotoxic activity of the extract and fractions of *E. graminea* leaves against the *Lemna minor*

Sample	Percentage Growth Inhibition (%) Concentrations (µg/mL)			
	10	100	1000	FI_{50} (µg/mL)
Extract	6.66 ± 0.33	19.99 ± 1.10	36.33 ± 3.30	>1000
Aqueous fraction	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	-
Chloroform fraction	23.33 ± 0.57	66.50 ± 1.18	96.67 ± 6.30	81.03
Paraquat	44.8 ± 0.1	78.5 ± 3.10	100 ± 0.0	25.15
Distilled water (-ve control)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	-

Values are expressed as the mean ± SEM of three independent observations.

Table 5: Sensitivity of the cervical cancer cell-lines (Hela) to the extract and fractions of *E. graminea*

Extracts	Concentration (µg/mL)	% Growth inhibition/ cytotoxicity	GI_{50} (µg/mL)	LC_{50} (µg/mL)	TGI (µg/mL)
Extract	1	0.00 ± 0.00			
	10	+11.48 ± 2.00			
	50	+11.48 ± 2.00	>250	>250	>250
	100	+11.48 ± 2.00			
	200	+11.48 ± 2.00			
	250	+11.48 ± 2.00			
Aqueous fraction	100	>100	>100	>100	>100
Chloroform fraction	1.0	+45.62± 6.44			
	25	+87.01± 10.51	68.0	72.54	54.57
	50	-9.00 ± 2.20			
	75	-22.00 ± 3.12			
	100	-52.00 ± 3.00			
Doxorubicin	0.01	-2.50± 0.01			
	0.1	-11.35 ± 2.50			
	0.5	-32.00 ± 1.98	0.17	6.12	8.72
	5.0	–85.50 ± 4.17			
	10.0	–87.00 ± 3.20			

Hela control absorbance at 545 nm = 3.0± 0.1 Each value reflects a Mean ± SEM percentage of triplicate experiments in comparison with control. Growth inhibition = + and cytotoxicity = –

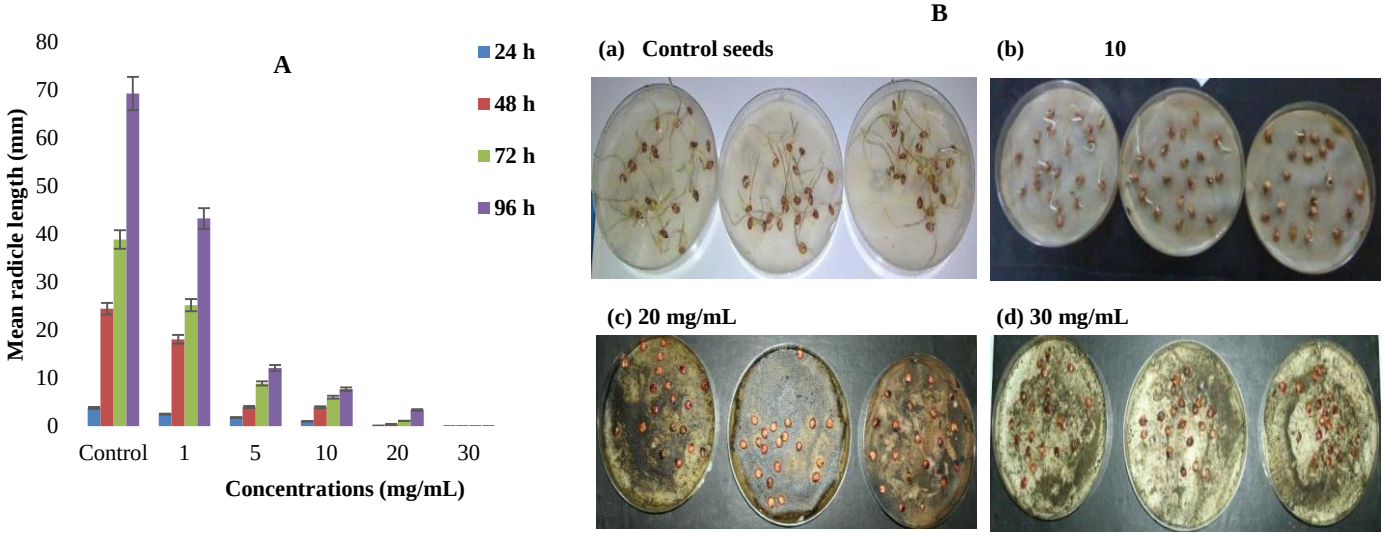

Figure 1: "**A**" - Graphical representation of effects of the extract on radicle length of *S. bicolor*; "**B**"- Plates showing the effects of the extract on radicle length of *S. bicolor* at different concentrations

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

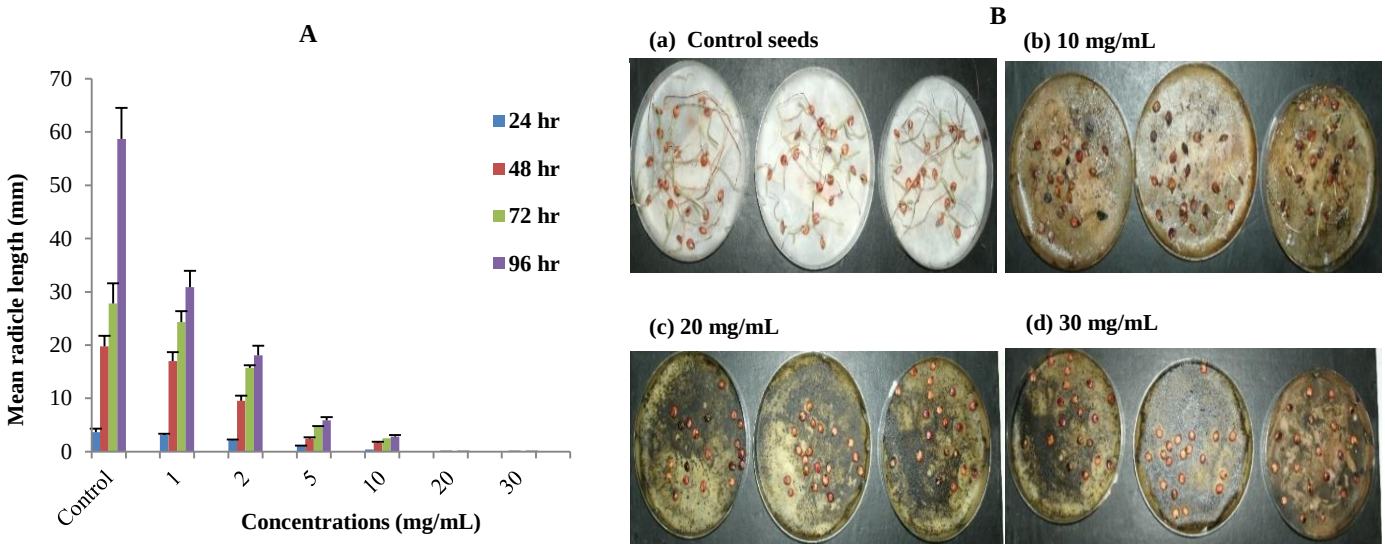

Figure 2: "A"- Column chart showing the antiproliferative effect of the chloroform fraction of *E. graminea* on the radicle length of guinea corn. "**B**"-Plates showing the effects of the chloroform fraction on radicle length of *S. bicolor* at different concentrations. Values are Mean ± S.E.M, n = 20.

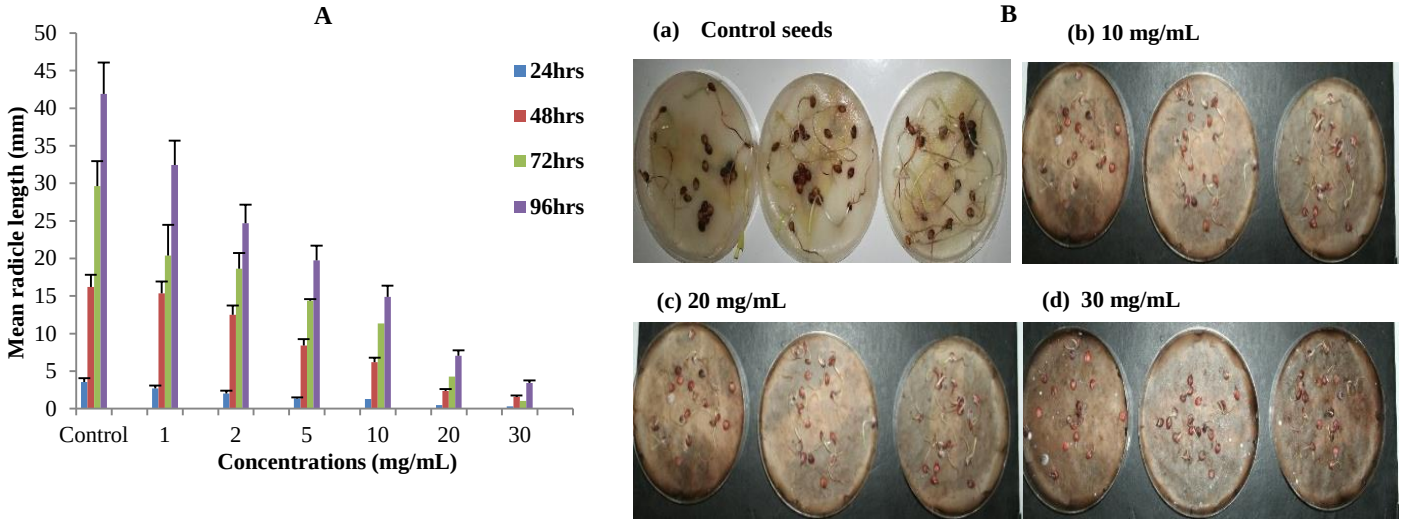

Figure 3: "A"- Effect of the aqueous fraction of *E. graminea* on the radicle length of guinea corn. Values are Mean ± S.E.M, n = 10. **"B"-** Plates showing the effects of the aqueous fraction on radicle length of *S. bicolor* at different concentrations.

Conclusion

This work has demonstrated the cytotoxicity, phytotoxicity and antiproliferative potentials of leaves of *E. graminea*. However, the biological potential of this plant was observed more in its chloroform fraction which recorded higher cytotoxic and phytotoxic activities in all the assays. The antiproliferative assay of medicinal plant-derived medications have had great impact in the antitumor sector and has led to the discovery of drugs such as Vinblastine, Vincristine, Taxol, etc.[26] It is our hope that further evaluation of this active chloroform fraction against other cancer cell lines could lead to the isolation of active principles which might serves as lead in anticancer drug discovery.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgements

We express our appreciation to the management and employees of the international Center for Chemical and Biological Sciences (ICCBS), the University of Karachi, Pakistan, for supporting and assisting in the carrying out part of this research work at the Institute.

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