Ethnobotanical Leaflets 13:832-39, 2009.

Evaluation of Cytotoxic Potential of *Acorus calamus* Rhizome

Rajkumar V, Gunjan Guha, Ashok Kumar R* and Lazar Mathew

School of Biotechnology, Chemical and Biomedical Engineering
VIT University, Vellore – 632 014
*Corresponding author Email: rashokkumar@vit.ac.in

Issued July 01 2009

Abstract

The present work evaluated the cytotoxicity of methanolic and aqueous extracts of rhizome of *Acorus calamus* Linn. which is a well known medicinal plant used in traditional treatment. Both extracts were found to be cytotoxic as determined by *Allium cepa* root tip assay and XTT assay in MDA-MB-435S and Hep3B cell lines. Results of the study indicate that the plant possesses anti-tumor properties and may serve as a potential source for investigation and development of anti-cancer drugs.

Key words: Cytotoxicity; *Allium cepa*; XTT; Anti-cancer, MDA-MB-435, Hep3B.

Introduction

A wide variety of secondary metabolites obtained from plants are tested for their ability to treat cancer. Various anti-cancer drugs from plants are known to be effective against proliferating cells. They exhibit cytotoxic effects either by damaging DNA or by blocking the formation of mitotic spindle during stages of cell division (Gali-Muhtasib and Bakkar, 2002). However most of the cytotoxic drugs exhibit side effects, and hence, there is a need for drugs that are efficient and have less side effects (Powis, 1983). The use of plant products in the treatment of cancer has been of recent interest (Bauer, 2000). Many drugs that are used for cancer treatment are at present obtained from plant sources. These include the well known vinca alkaloids, vincristine and vinblastin, isolated from *Catharanthus roseus*, etoposide and teniposide, derivatives of epipodophyllotoxin, isolated from *Podophyllum*, and several others (Cragg *et al.*, 1993, 1994; Wang, 1998).

The present study aims at evaluating the cytotoxicity of *Acorus calamus* Linn. rhizome. We tested cytotoxicity and anti-proliferative activity of aqueous and methanolic extracts by the *Allium cepa* root tip assay and XTT assay on MDA-MB-435S (human breast carcinoma) and Hep3B (liver carcinoma) cell lines.

Methods

Chemicals

Vincristine sulfate was purchased from Sigma-Aldrich. Colchicine, L-15 (Leibovitz) medium with L-glutamine, Minimal

Essential Medium (MEM) Eagle with Earle's salt, NEAA and L-glutamine and phenazine methosulphate (PMS) were purchased from Hi-Media (Mumbai, India). MDA-MB-435S (human breast carcinoma) and Hep3B (human liver carcinoma) cell lines were obtained from National Center for Cell Science (NCCS), Pune, India. All other chemicals and solvents were of the highest commercial grade and used without further purification.

Collection of plants

Acorus calamus rhizomes were obtained from its natural habitat in Coimbatore region, Tamil Nadu, India in the month of December, 2007. They were shade-dried, powdered, sieved and stored prior to further use. Voucher specimens were maintained in our laboratory for future reference.

Extraction

Rhizome powder was extracted sequentially with methanol and water using a Soxhlet apparatus and evaporated to dryness in vacuum at 60°C in a rotary evaporator (Buchi, Switzerland) (Saeedeh *et al.*, 2007). The dried extracts in suitable concentrations were tested for cytotoxicity.

***Allium cepa* root tip assay**

Allium cepa root tip meristems have been widely used for the testing of cytotoxicity and anti-mitotic activity of various compounds (Grant *et al*., 1981). *A. cepa* root tip assay was introduced by Levan in 1938 and was later proposed as a standard method to study genotoxicity (Fiskesjo, 1985). 100 µg/ml, 200 µg/ml, 500 µg/ml and 1000 µg/ml of the extracts were used for the assay. Bases of onion bulbs were suspended in extract solutions. At the end of exposure periods (48 and 72 h), root lengths were measured (in cms) with a ruler. Onions grown on double distilled water (without any extract) served as the negative control. Vincristine and colchicine were employed as positive controls (data not shown).

Mitotic index calculation

Mitotic index was calculated as described by Bloch *et al.* (1967). Roots were excised at 48 and 72 h, and suspended in a fixative (ethanol: acetic acid = 3:1) for 10 min. They were then washed with distilled water, hydrolyzed at 60 °C in 1N HCl for 15 min, and stained with 0.5% hematoxylin stain for 10 min. Stained root tips were excised and squashed on a clean glass slide with a drop of 45% acetic acid and examined under microscope. In all the slides, 400-500 cells were counted to determine the number of cells in interphase and dividing phase (Sehgal *et al.*, 2006). Mitotic index was calculated by using the formula:

$$\text{Mitotic index percentage (\%MI)} = \frac{\text{Total number of cells in mitosis}}{\text{Total number of cells counted}} \times 100$$

Analysis of cytotoxicity: XTT assay

XTT assay was performed on MDA-MB-435S (grown in L-15 medium) and Hep3B (grown in MEM medium) cell lines as described by Weislow *et al*. (1989). 6×10^3 cells were seeded on 96-well plates and the cells were supplemented with 200 µl of the respective culture medium for a period of 24 h. The media were then substituted by 200 µl of fresh media containing varying concentrations of the extracts (15.625 µg/ml, 31.25 µg/ml,

62.5 µg/ml and 125 µg/ml). The plates were incubated at 37 °C for 24 h, after which, media were removed and fresh media were added. 50 µl of XTT reagent prepared in medium (0.6 mg/ml) containing 25 µM of PMS was then added to all the wells and the plates were incubated in dark humid conditions at 37 °C for 4 h. After incubation, the orange colored complex formed was read at 450 nm using a Dynex Opsys MR^{TM} Microplate Reader (Dynex Technologies, VA, USA) with a 630 nm reference filter. Wells containing cells without extract treatments served as the control. Wells containing only culture medium and XTT reagent served as the blank. Percentage cytotoxicity of the extracts was calculated by using the formula:

$$\% \text{ Cytotoxicity} = \frac{(\text{OD of control} - \text{OD of treated cells})}{\text{OD of control}} X\ 100$$

Statistical analysis

All data were recorded as mean ± standard deviation of triplicate measurements. Significant differences among treatment means were determined by ANOVA at $P<0.05$. MATLAB ver. 7.0 (Natick, MA, USA) and Microsoft Excel 2007 (Roselle, IL, USA) were used for the statistical evaluations.

RESULTS

Extract yield

A. calamus rhizome powder was extracted sequentially with methanol and water as solvents. 50 g of rhizome powder yielded 16.4 g of crude methanolic extract and 6.91 g of crude aqueous extract.

Root length and mitotic Index of *Allium cepa*

The inhibitory effects of the extracts were evaluated on the growth of *Allium cepa* root meristem. Root lengths and % mitotic index (in root tip cells) in control group and treated groups at 48 h and 72 h are given in Table 1.

Table 1: Root length and % mitotic index of root tip cells of *Allium cepa* at 48 h and 72 h of exposure to different concentrations of aqueous and methanolic extracts of *A. calamus*.

Name of the extract	48 hrs		72 hrs	
	Root length (in cms)[a]	% MI	Root length (in cms)[a]	% MI
Control	1.87 ± 0.35 (n=29)	72.68	2.82 ± 0.92 (n=38)	69.72
Acorus calamus aqueous extract				
100 µg/ml	1.82 ± 0.61 (n=15)	70.42	2.32 ± 0.64 (n=22)	67.59

200 µg/ml	1.55 ± 0.91 (n=8)	65.02	1.96 ± 0.50 (n=19)	60.36
500 µg/ml	0.97 ± 0.24 (n=18)	50.18	1.09 ± 0.15 (n=22)	58.04
1000 µg/ml	0.62 ± 0.29 (n=20)	37.62	0.75 ± 0.16 (n=11)	23.63
Acorus calamus methanolic extract				
100 µg/ml	1.88 ± 0.35 (n=22)	67.92	1.86 ± 0.33 (n=25)	66.02
200 µg/ml	1.40 ± 0.2 (n=31)	64.02	1.25 ± 0.78 (n=20)	60.61
500 µg/ml	0.92 ± 0.29 (n=21)	56.12	1.02 ± 0.06 (n=15)	58.72
1000 µg/ml	0.75 ± 0.28 (n=16)	34.62	0.89 ± 0.25 (n=20)	35.49

[a] mean root length ± SD at 95% confidence interval

XTT assay

Table 2 presents the IC_{50} values for the aqueous and methanolic extracts of *A. calamus* in MDA-MB-435S and Hep3B cell lines. Although the magnitude of cytotoxicity varied among the two cell lines, considerable cytotoxicity was demonstrated in both cell lines, thereby indicating the presence of anti-cancer metabolites.

Table 2: IC_{50} values of aqueous and methanolic extracts of *A. calamus* in MDA-MB-435S and Hep3B cell lines as determined in XTT assay.

	MDA-MB-435S	Hep3B
Aqueous extract	63.65 ± 8.30 µg/ml	85.22 ± 11.40 µg/ml
Methanolic extract	13.71 ± 6.66 µg/ml	32.74 ± 4.55 µg/ml

DISCUSSION

In this study, both extracts of *A. calamus* rhizome showed cytotoxicity. The results indicate that these plant products might act against development of cancer. There was a significant concentration and time dependent decrease ($P<0.05$) in *A. cepa* root length along with a simultaneous decline in percentage mitotic index of root tip cells.

The extracts demonstrated congruous trend of cytotoxicity in all test models. Correlations between results from all

test systems were analyzed ($P<0.05$). Methanolic extracts showed significant positive correlation between cytotoxicity demonstrated in MDA-MB-435S and *A. cepa* root tip cells for both 48 h ($r^2 = 0.99276$) and 72 h ($r^2 = 0.9901$) treatments; as well as cytotoxicity between Hep3B and *A. cepa* root tip cells [$r^2 = 0.982278$ (48 h); $r^2 = 0.976605$ (72 h)]. Aqueous extract, on the other hand, did not demonstrate any significant correlation with respect to cytotoxicity between *A. cepa* root tip cells and either of the cell lines. Furthermore, cytotoxicity caused in MDA-MB-435S and in Hep3B cell lines by the methanolic extract have a significant positive mutual correlation ($r^2 = 0.986297694$), which is lacking in case of the aqueous extract. These differences observed in correlation might be due to diverse components with differential activities towards various cell types being eluted in the solvents employed.

According to the American National Cancer Institute, the IC_{50} limit to consider a crude extract promising for further purification is lower than 30 µg/ml (Suffness and Pezzuto, 1990). IC_{50} values of the methanolic extract for both cell lines are well within this limit. These results strongly prove that methanolic extract of *A. calamus* has a strong and consistent anti-proliferative effect on plant tissue cells and animal cell lines.

Conclusion

The study concludes that rhizome of *Acorus calamus* might be considered as a potential source of metabolites which could be developed as precursors for anti-cancer drugs. Isolation and purification of these active compounds are in prospect.

Acknowledgement

We are thankful to the management of VIT University, Vellore, Tamil Nadu, India, for providing the necessary facilities and infrastructure for the successful accomplishment of this work.

References

1. Arabshahi-Delouee, S. Urooj, A. 2007. Antioxidant properties of various solvent extracts of mulberry (*Morus indica* L.) leaves, *Food Chem* 102: 1233–1240.

2. Bauer, B. A. 2000, *Mayo Clin. Proc* 75(8): 835.

3. Bloch, D. P, Macquigg, R. A, Brack, S. D and Wu, J. R. 1967. The syntheses of deoxyribonucleic Acid and histone in the onion root meristem, *J. Cell Biol* 88: 451-467.

4. Cragg G.M, Boyd M.R, Cardellina J.H, Newman D.J, Snader K.M, McCloud T.G (1994). Ethnobotany and drug discovery experience of the US National Cancer Institute: In: Chadwick D.J, Marsh J (Eds.), *Ciba foundation "Ethnobotany and the search for new drugs" symposium*, 185. Wiley and Sons, Chichester, UK, pp. 178-196.

5. Cragg G.M, Schepartz S.A, Stuffness M, Grever M.R (1993). The taxol supply crisis. New NCI policies for handling the large-scale production of novel natural product anticancer and anti-HIV agents. *J. Nat. Prod* 56: 1657-1668.

6. Fiskesjo G., 1985. The Allium test as a standard in environmental monitoring. *Hereditas* 102, 99-112.

7. Gali-Muhtasib H and Bakkar N (2002). Modulating cell cycle: current applications and future prospects for future drug development. *Curr. Cancer Drug Targets* 2: 1-17.

8. Grant W.F, Zinov Eva-Stahevitch A.E and Zura K.D (1981). Plant genetic test systems for the detection of chemical mutagens. In: H. F. Spich and R. H. C. San (Eds.), *Short-term Tests for Chemical Carcinogens*, Springer-Verlag, New York, 200-216.

9. Levan A (1938). The effect of colchicine on root mitoses in Allium. *Hereditas* 24: 471-486.

10. Powis G (1983). Dose-dependent metabolism, therapeutic effect, and toxicity of anticancer drugs in man. *Drug Metab. Rev* 14: 1146-1163.

11. Sehgal R, Roy S, Kumar V.L (2006). Evaluation of cytotoxic potential of latex of Calotropis procera and Podophyllotoxin in Allum cepa root model. *Biocell* 30(1): 9-13.

12. Stuffness M and Pezzuto J.M (1990). Assays related to cancer drug discovery. In: Hostettmann K (Ed.), *Methods in Plant Biochemistry: Assays for Bioactivity*, 6. Academis Press, London, pp. 71-133.

13. Wang H.K (1998). Plant-derived anticancer agents currently in clinical use or clinical trials. *The Investigation Drugs Journal* 1: 92-102.

14. Weislow O.S, Kiser R, Fine D.L, Bader J, Shoemaker R.H and Boyd M.R (1989). New soluble-formazan assay for HIV-1 cytopathic effects: application to high-flux screening of synthetic and natural products for AIDS-antiviral activity. *J. Natl. Cancer Inst* 81: 577–586.