ANTICANCER ACTIVITY OF THE ETHANOLIC EXTRACTS OF *AGAVE AMERICANA* LEAVES.

Ketan V. Khade, Harikesh Dubey*, Chandrashekhar R. Tenpe,
Pramod G. Yeole, Angad M. Patole

Institute of Pharmaceutical Education and Research (IPER), Wardha
(MS) 442 001, India.

E-mail: dubey.harikesh@gmail.com

Summary

The present study was designed to determine the anticancer activity of the ethanolic extract of leaves of *Agave americana* L. (Agavaceae) using three reported methods. For brine shrimp lethality bioassay ten nauplii were placed in test tube filled with 5 ml total volume of artificial sea water and different concentrations (100, 500, 1000, 2000, 5000 µg/ml) of ethanolic extract of *Agave americana* leaves in a set of test tubes per dose. After 24 hours, live nauplii were counted and LC_{50} value was estimated. In *Allium cepa* root meristem model, onion bulbs were suspended inside 100 ml beakers at different concentration (1 mg/ml and 10 mg/ml) of extract. The percentage root growth inhibition after treating with ethanolic extract at 48 and 96 hrs was determined. MTT assay have been utilised to measure the antitumor activity of ethanolic extract of *A. americana* leaves by using PA-1 human cell line of ovarian teratocarcinoma. Brine shrimp lethality bioassay (LC_{50}= 923.10µg/ml), *Allium cepa* root meristem model and MTT assay (IC_{50} =0.01µg/ml) showed potent cytotoxic and anticancer activity of ethanolic extract of *A. americana* leaves. The ethanolic extract of *A. americana* leaves has a cytotoxic and antitumor activity. Therefore, this plant has potential to be utilized for the development of novel anticancer drug leads.

Keywords: MTT assay (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, Brine shrimp lethality bioassay (BSLB), teratocarcinoma, PA 1 cell line.

Introduction

Over the past decade herbal medicine have been accepted universally, hence medicinal plants continue to play an important role in healthcare system of a large number of world's population. In fact there are several medicinal plants all over the world which are being used traditionally in the prevention and treatment of cancer. Plant derived compounds have played an important role in the development of several clinically useful anti-cancer agents (1).

Plant traditionally used for warts, cancerous ulcer, putrid tumours, sacrofula, scabies, as a diuretic, emmenagogue and insecticide. Various parts of this plant are used for ascites, dropsy, anasarca, veneral sores, syphilis, dysentery and as a diaphoretic (2, 3, 4). *Agave* sap used in wound healing, tincture used in diarrhoea and dysentery, juice used in appetite, constipation, scurvy stranguary, swollen and bleeding gums, liver disease, ulcer and inflammatory condition, used in manufacturing of steroid hormone synthesis (4, 5).

Our study demonstrated the cytotoxic activity of ethanolic extract of leaves of *A. americana* by reported models viz Brine shrimp lethality bioassay (BSLB), *Allium cepa* root tip meristem model and MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. *Artemia salina L. (Artemiidae)*, the brine shrimp, is an invertebrate of the fauna of saline aquatic and marine ecosystems. It can be used in laboratory bioassay to determine toxicity through the estimation of median lethal concentration (LC_{50} values) which has been reported for series of toxins and plant extracts. Several naturally extracted products which had $LC_{50} < 1000\mu g/ml$ using BSLB were known to contain physiologically active principles (6, 7).

Allium cepa root tip meristem have been widely used for the evaluation of cytotoxicity, anti-mitotic (8, 9), genotoxicity (10, 11), antimutagenic (12) and antioxidant activity (13, 14) by employing the growing roots of *Allium cepa*. The MTT assay, developed by Mosmann, to measure cell proliferation and activation, is one of the most used methods for preliminary screening of different substances in cell cultures (15). This technique measures the ability of living cells to reduce the tetrazolium salt, MTT, to formazan by NADH reductase and other enzymes which can be measured spectrophotometrically. It is well established that there is a linear

relationship between the number of live cells and the amount of formazan produced, thus validating this methodology to measure cellular metabolic activity. MTT enter the cell by endocytosis and is converted to fromazan, the amount of formazan reflects the reductive potential of the cytoplasm and the cell viability (16).

Materials and Methods

The A. *americana* leaves were collected from Nagpur District, Maharashtra, India in the month of August 2008. The plant was identified and authenticated by Dr. Alka Chaturvedi, "Post graduate Teaching Department of Botany, Rashtrasant Tukadoji Maharaj, Nagpur University, Nagpur. A voucher specimen (No.9402) was deposited with the, "Post graduate Teaching Department of Botany, RTM Nagpur University, Nagpur. All the chemicals used for experimental purpose were of laboratory grade. Brine shrimp (*Artemia Salina*) eggs were purchased from Matsyakanya Aquarium, 480/58, Cannada Corner, Nasik-5, Maharashtra and the standards drug i.e. Cyclophoshpamide and Paclitaxel were provided as gift sample by VHB life sciences Rudrapur, Uttaranchal, India.

The shade dried and powdered leaves of *A. americana* were subjected to successive extraction in a soxhlet apparatus with petroleum ether, chloroform, ethanol and finally macerated with water so as to get respective extracts. All extracts were individually filtered, through Whatmann filter paper # 42 and evaporated to dryness at 50ºC in oven. The ratio of powder to solvent was 10:100. The extracts were then stored in desiccators till further use. Extractive values for ethanolic extract was found to be 14.7 %. Ethanolic extract shows maximum potency as compare to other extracts.

In vitro cytotoxic activity

Brine Shrimp Lethality Bioassay

Brine shrimp (*Artemia salina*) eggs (150 mg) were hatched in a conical shaped vessel (1L) filled with sterile artificial sea water under constant aeration for 72 hrs. To avoid risk of larvae death because of reduction in pH during incubation, pH was adjusted to 8.5 (17, 18, 19). 15ml of yeast solution 0.06% was added to vessel for every litre of salt water after 48 hrs in order to feed larvae. It

takes about 72 hours for hatching, active nauplii free from egg shells were collected and used for the assay (20, 21).

Ten active nauplii were placed in test tube containing sample, filled with 5 ml total volume of artificial sea water. Experiment was conducted using set of three test tubes per concentration of extract (100, 500, 1000, 2000, 5000 µg/ml), control (vehicle) and standard drug (Cyclophosphamide), after 24 hours, live nauplii were counted and LC_{50} value was estimated. The percentage lethality was determined by comprising the mean surviving larvae of the test and control tubes. LC_{50} values were obtained from, concentration verses percentage lethality by using statistical method of Finney's probit analysis (19, 20, 22).

Allium cepa Root Tip Meristem Model

Locally available onion bulbs (*Allium cepa* 50 ± 10 g) were grown in the dark over 100 ml tap water at ambient temperature until the roots have grown to approximately 2-3 cm length. The base of each of the bulbs were suspended on the extract inside 100 ml beakers, root length (newly appearing roots not included) and root number at 0, 48, 96 hrs for each concentration of extract and control was measured. The percentage root growth inhibition after treating with ethanolic extract at 48 and 96 hrs was determined. Cyclophosphamide (standard) as well as extract of leaves was used at 1 mg/ml and 10 mg/ml concentration (8).

MTT Assay

Effect of ethanolic extract of *A. americana* leaves on cell proliferation of PA-1 human cell line of ovarian teratocarcinoma was carried out by MTT assay. Doxorubicin is used as standard anticancer drug MTT assay. Monolayer cultures were incubated up to 96 hours in microtitration plates in a range of drug different concentrations of ethanolic extracts of *A. americana* leaves (Table-6). The extract was removed and feeded the plates daily for 2-3 PDTs, then again plates were feeded and MTT added to each well. Incubate the plate in dark and then the medium and MTT was removed. The water-insoluble MTT-formazan crystals were dissolved in DMSO, buffer was added to adjust the final pH, and recorded the absorbance (23, 24).

Results

Brine shrimp lethality activity of ethanolic extracts of A. *Americana* leaves, was found to be directly proportional to the concentration of the extract. The ethanolic extract of *A. americana* leaves have shown the LC_{50} value of 923.10µg/ml (Table 3) and LC_{50} value of cyclophosphamide was found to be 794.43µg/ml (Table 2). While there was no Brine shrimp lethality activity shows in control (Table 1). Results show that ethanolic extract of *A. Americana* has potent cytotoxic effect which is similar to standard drug. The LC_{50} value for extract of *A. americana* leaves in our study was found to be lower than 1000µg/ml.

Table 1:- Observations of Average mortality for Control

Control	Concentration (µg/ml)	No. of test Tube	No. of Shrimp Tested	Average Mortality After (24.hrs)	Percent Mortality After (24.hrs)
Without drug	----	1-a	10	00	00
		1-b	10		
		1-c	10		

Table 2: Observations of Average mortality and LC_{50} value (Cyclophosphamide)

S. No.	Concentration (µg/ml)	No. of test Tube	No. of Shrimp test	Average Mortaliy After 24hrs	Percent Average mortality	LC_{50} (µg/ml)
1	100	1-a	10	01	10	794.43
		1-b	10			
		1-c	10			
2	500	2-a	10	02	20	
		2-b	10			
		2-c	10			
3	1000	3-a	10	08	80	
		3-b	10			
		3-c	10			
4	2000	4-a	10	09	90	
		4-b	10			

		4-c	10			
5	5000	5-a	10	10	100	
		5-b	10			
		5-c	10			

Cyclophosphamide is used currently as standard anticancer drug (Positive control). In our study as expected, LC_{50} value for the Cyclophosphamide is lesser than 1000 µg/ml. LC_{50} value has been estimated (after 24 hours, live nauplii were counted and analyzed) statistically by Finney's Probit Analysis Method and was found to be 794.43. Hence, Cyclophosphamide is bioactive by BSLB Model with LC_{50} = 794.43

Table 3: Observations for Average mortality and LC_{50} value of Ethanol Extract of leaves:

S. No.	Concentration (µg/ml)	No. of test Tube	No. of Shrimp test	Average Mortality After 24hrs	Percent Average mortality	LC_{50} (µg/ml)
1	100	1-a	10	01	10	923.10
		1-b	10			
		1-c	10			
2	500	2-a	10	03	30	
		2-b	10			
		2-c	10			
3	1000	3-a	10	06	60	
		3-b	10			
		3-c	10			
4	2000	4-a	10	09	90	
		4-b	10			
		4-c	10			
5	5000	5-a	10	10	100	
		5-b	10			
		5-c	10			

The degree of lethality was found to be directly proportional to concentration of ethanolic extract. In this study LC_{50} value for the Ethanolic Extract lesser than 1000µg/ml. LC_{50} value has been estimated (after 24 hours, live nauplii were counted and analysed) statistically by Finney's Probit Analysis Method and was found to be 923.10 µg/ml. Hence, Ethanolic Extract is bioactive by BSLB method with LC_{50} = 923.10

The cytotoxic effect of ethanolic extract of *A. americana* leaves was also evaluated by using *Allium cepa* root tip meristems. In control group a progressive increase in root number and root length was observed. The root length in control group at 0, 48 and 96 h was 3.33±0.67 cm (n=16), 4.19±0.81 cm (n=22) and 4.63±1.10 cm (n=28) respectively (Table- 4). The extract of *A. americana* leaves produced dose and time dependent growth inhibition. Incubation of bulbs in different concentrations of extract and standard produced a growth retarding effect that was associated with a decrease in the root number (Fig.1). *Allium cepa* root tip meristem growth inhibition was highest with significance of ($p<0.01$) at the 10 mg/ml concentration after 96 hrs for ethanolic leaves extract. The root length after 0, 48 and 96 hrs with significance of ($p<0.01$) at 10 mg/ml was found to be 2.39$\pm$0.21 (n =17), 1.83$\pm$0.19 (n =12), 1.23$\pm$0.17(n =9) respectively (Table 4).

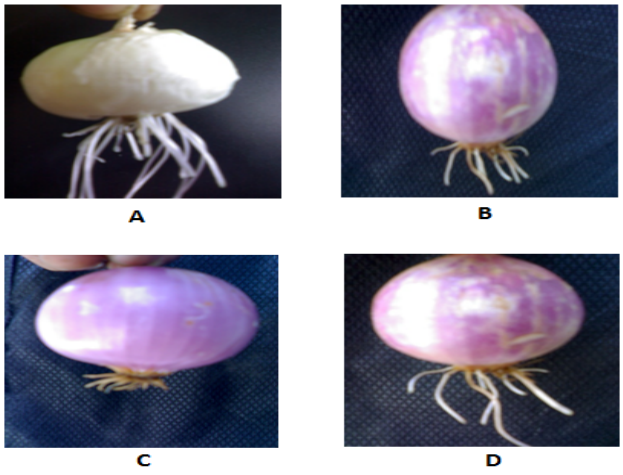

Fig. 1. *Allium cepa* bulbs showing the effect of different extracts of roots of *A. americana* on root length following 96 h of incubation.

(A) Control (B) Cyclophosphamide (C) Paclitaxel (D) Ethanol Extract,

Table 4: Observations for *Allium cepa* root length and root number attained following incubation with ethanolic extracts of *A. americana* leaves and standard drugs.

Groups	Concentration	Root length (cms)		
		(0) hr	(48) hr	(96) hr
Control	----------	3.33±0.67 (n=16)	4.19±0.81 (n=22)	4.63±1.10 (n=28)
Ethanol	1mg/ml	2.56±0.15 (n=18)	2.38±0.13 (n=14)	2.23±.21* (n=11)
	10mg/ml	2.39±0.21 (n=17)	1.83±0.19 (n=12)	1.23±0.17** (n=09)
Cyclophosp-hamide	1mg/ml	2.55±0.26 (n=13)	2.42±0.27 (n=17)	2.35±0.34* (n=21)
	10mg/ml	2.47±0.11 (n=14)	2.23±0.22 (n=12)	2.07 ± 0.26* (n=09)
Paclitaxel	0.05mg/ml	3.75±0.71 (n=15)	2.88±0.19 (n=12)	1.97±0.12** (n=08)
	0.5 mg/ml	2.74±0.63 (n=13)	1.85±0.13 (n=09)	1.07±0.14** (n=04)

* Indicates Significant One-way Analysis Variance of P value < 0.05 for root number (n) and root length.

** Indicates Significant One-way Analysis Variance of P value < 0.01 for root number (n) and root length.

Furthermore in our study, in vitro effect of ethanolic extracts of *A. americana* leaves on cell proliferation and growth was carried out by MTT assay using human cell line (Table 5). The results obtained from assay (Table 6 and 7) shows IC_{50} value of 0.01µg/ml (figure 2).

Table 5: Cytotoxicity analysis of *A.americana* leaves extracts against teratocarcinoma

Assay used :	MTT
Time of incubation	96 h
Cell Line	PA-1
Organism	*Homo sapiens* (human)
Organ	Ovary
Tissue	Epithelial
Disease	Teratocarcinoma
Derived from metastatic site	Ascites
Antigen expression	HLA A28, B12
Oncogene	N-ras + (activated)
DNA Profile (STR):	Amelogenin: X CSF1PO: 9,12 D13S317: 9,10 D16S539: 9,12 D5S818: 11 D7S820: 9 THO1: 7,9 TPOX: 11 vWA: 15,17
Isoenzymes:	G6PD, B
Age:	12 years
Gender:	Female
Comments:	The line was established from cells taken from ascetic fluid. The cells form tightly knit colonies, and differentiate to form embryoid bodies when cultured in low serum concentration or at low plating densities or when treated with 5-bromo-2'-deoxyuridine. The embryonic antigen PCC4 is expressed, but F9 is not detectable.

Table 6: % Inhibition at different concentration of ethanolic leaves extract against teratocarcinoma

Concentration (µg/ml)	% Inhibition
0.001	45.60
0.01	62.00
0.1	62.80
1	63.60
10	64.00

Table 7: IC_{50} values of respective extract of *A. americana* leaves

Name of Extract	IC_{50}	Status
Ethanol Extract of leaves	0.01 µg	Active
Standard doxorubicin	500Nm	Active

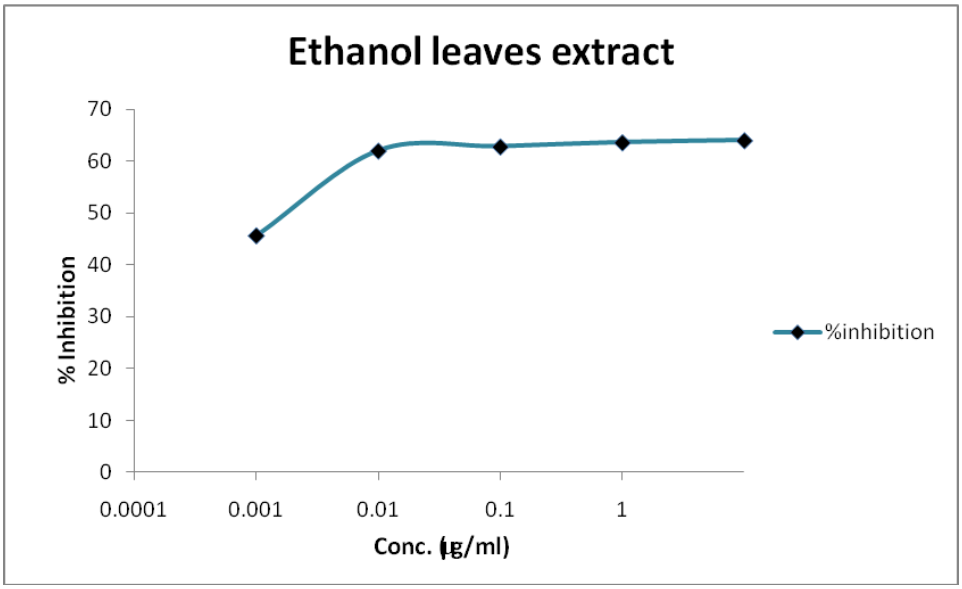

Fig. 2. % Inhibition at different concentration of extract against teratocarcinoma

Discussion

In the present study BSLB has been used. It is simple bioassay useful for screening large number of extracts in the drug discovery process and has been used in number of previous studies (25, 26, 27, 28). BSLB and other *in vivo* lethality test have been successively employed for bioassay-guide fractionation of active cytotoxic and antitumor agents (29). The technique has been used to identify over 300 novel antitumor and pesticidal natural products. BSLB found to have a positive correlation with human nasopharyngeal carcinoma cytotoxicity (30, 31). BSLB also has been reported to be useful in predicting other biological activities such as phototoxicity, trypanocidal, enzyme inhibition, ion regulation activities (32, 33)

and hepatotoxicity (34). The brine shrimp lethality activity of ethanolic extracts of *A. americana* leaves, after testing the extract with brine shrimp (Table 3) shows that there is linear dose-effect relationship between extract concentrations and LC_{50} value. The degree of lethality was found to be directly proportional to the concentration of the extract. The ethanolic extract of *A. americana* leaves has shown the LC_{50} value of 923.10µg/ml while LC_{50} value of Cyclophosphamide was found to be 794.43µg/ml. Results showed that ethanolic extract of *A. americana* leaves has potent cytotoxic effect by using BSLB which is comparable to that of standard drug (Cyclophosphamide). LC_{50} lower than1000µg/ml in the BSLB is considered to biologically active. The LC_{50} value for extract of *A. americana* leaves in our study was found to be lower than 1000µg/ml. Hence, ethanolic extract of leaves is bioactive by BSLB method with LC_{50} 923.10 and it can have pharmacological activity. Several naturally extracted products which had LC_{50} < 1000µg/ml using BSLB were known to contain physiologically active principles (6, 7).

Allium cepa root tip meristem model, a standardized test for cytoxicity monitoring and has been used by various authors (12, 35, 36). In our study extract of *A. americana* leaves produced dose and time dependent growth inhibition. Incubation of bulbs in different concentrations of extracts of *A. americana* leaves produced a growth retarding effect that was associated with a decrease in the root number (Fig.1). *Allium cepa* root tip meristem growth inhibition was highest with significance of ($p<0.01$) at the 10 mg/ml concentration after 96 hrs for extract. Ethanolic extract of *A. Americana* leaves and cyclophosphamide arrested the root growth. Its cytotoxic effect was also evident in the form of shortening and decaying of roots. However, the root number did not increase any further at 10 mg/ml concentration. Cytotoxic effect of ethanolic extract of *A. Americana* leaves by using *Allium cepa* root tip meristem model was comparable to that of Cyclophosphamide and Paclitaxel.

Furthermore in our study, cell proliferation activity of ethanolic extract of A. *Americana* leaves was carried out by MTT assay using human cell line. MTT assay still remains an adequate method for in vitro study of the cell viability and proliferative activity of the cell (37, 38). Measurement of cell viability and proliferation forms is used as basis for this in vitro assay. We estimated the effect of

extract on the growth of cell in vitro. The results obtained from assay (Table 6 and 7) showed IC_{50} value of 0.01µg/ml. It confirmed cytotoxic activity of ethanolic extract of *A. americana* leaves. The MTT assay generally shows a good correlation with other viability tests and in vivo results (23). The MTT bioassay is a rapid, versatile, quantitative, and highly reproducible colorimetric assay. It also have been used for mycotoxins screening (15), cytotoxic activities of natural killer cells (24), neurotoxicity studies of Aβ peptides (37) and in vitro drug interactions studies (39). The MTT assay has been used as an alternative to the radioactive techniques (24).

In the present study, three different methods were used for evaluation of cytotoxic activity which have different sensitivity confirms the cytotoxic activity of *A. americana* leaves extract. BSLB is in vivo method and shows positive correlation with human nasopharyngeal carcinoma. Human carcinoma cell line have been utilised in MTT assay to evaluate cytotoxic activity. In *Allium cepa* root tip meristems model, root tips which are growing have been used, cytotoxic effect was also evident in the form of shortening and decaying of roots both in extract and standard (paclitaxel). The standard drug used paclitaxel is known mitotic inhibitor, probably *A. americana* leaves extract might have mitotic inhibitory activity further study is required to clear this aspect.

Thus, our study demonstrates that ethanolic extract of *A. americana* leaves exhibits potent cytotoxic property by all three methods used which comparable to standard anticancer drugs.

Conclusion

The results of the our study revealed that the ethanol extract of *A. americana* has a potential cytotoxic and antitumor activity, further study is required to establish the antitumor activity of this plant's isolated compounds in vivo and in vitro with different other human cell lines.

Acknowledgement

We would like to thank Deshpande Laboratories, Bhopal (India), for experimental analysis (MTT Assay).

References

1. Madhuri S, Pandey G. Some anticancer plants of foreign origin. *current sci* 2009; 96: 6.
2. Asolkar LV, Kakkar KK, Charke OJ. *Second supplement to glossary of Indian medicinal plants with active principles*. New Delhi, Publication and information directorate, 1992: 29.
3. Kirtikar, KR, Basu BD. Indian Medicinal Plant. Dehradun: International book distributors, 1987: 2466-68.
4. Tinto WF, Simmons-Boyce JL, McLean S, et al. Constituents of *Agave americana* and *Agave barbadensis*. *Fitoterapia* 2005; 76, 594-7.
5. Khare CP. Encyclopedia of Indian medicinal plant. Berlin Heidelberg: Springer-verlag, 2004: 33-34.
6. Mathews RS. *Artemia salina* as a test organism for measuring superoxide-mediated toxicity. *Free Radia Biol Med* 1995; 18(5): 919-22.
7. Ibrahim AMM, Mostafa MH, El-Masry MH, et al. Active biological materials inhibiting tumor intiation extracted from marine alga. Egypt J Aquatic Res 2005; 31(1): 146-155.
8. Sehgal R, Roy S, Kumar VL. Evaluation of cytotoxic potential of latex of *Calotropis procera* and podophyllotoxin in *Allium cepa* root model. *Biocell* 2006; 30(1): 9-13.
9. Akinboro A, Bakare A. Cytotoxic and genotoxic effects of aqueous extracts of five medicinal plants on *Allium cepa Linn. J Ethnopharmacol* 2007; 112: 470-75.
10. Oyedare B, Bakare A, Akinboro A. Genotoxicity assessment of water extracts of *Ocimum gratissimum, Morinda lucida* and *Citrus medica* using the *Allium cepa* assay. *Bol Latinoam Caribe Plant Med Aromat* 2009; 8(2): 97-103.
11. Seth C, Misra V, Chauhan L, et al. Genotoxicity of cadmium on root meristem cells of *Allium cepa*: cytogenetic and Comet assay approach. *Ecotoxicol and Environ Safety* 2008; 71: 711-16.

12. Sultan A, Celik T. β Genotoxic and Antimutagenic Effects of *Capparis spinosa* L. on the *Allium cepa* L. Root Tip Meristem Cells. *Caryologia* 2009; 62(2): 114-23.
13. Rose P, Widder S, Looft J, et al. Inhibition of peroxynitrite-mediated cellular toxicity, tyrosine nitration and α1–antiproteinase inactivation by 3-mercapto-2-methylpentan-1-ol, a novel compound isolated from *Allium cepa*. *Biochem and Biophys Res Comm* 2003; 302: 397-402.
14. Achary V, Jena S, Panda K, et al. Aluminium induced oxidative stress and DNA damage in root cells of *Allium cepa* L. *Ecotoxicol and Environ Safety* 2008; 70:300-310.
15. Cetin Y, Bullerman LB. Cytotoxicity of Fusarium mycotoxins to mammalian cell cultures as determined by the MTT bioassay. *Food and Chem Toxicol* 2005; 43:755-64.
16. Liu Y, Schubert D. Cytotoxic amyloid peptides inhibit cellular 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) reduction by enhancing MTT formazan exocytosis. *J. Neurochem* 1997; 69 (6): 2285-93.
17. Steenkamp V, Eloff JN. Evaluation of *Athrixia bush* tea for ctotoxicity, antioxidant activity, caffeine content and presence of pyrrolizidine alkaloids. *J Ethnopharmacol* 2007; 110: 16-22.
18. Kapadia GJ, Nishino H. Chemopreventive effect of Resveratrol, Sesamol, Sesame oil and Sunflower oil in the Epstein-Barr virus early antigen activation assay and the mouse skin two-satge carcinogenesis. *Pharmacological Reas* 2002; 45(6): 499-505.
19. Obuotor EM, Onajobi FD. Preliminary evaluation of cytotoxic properties of *Raphia hookeri* fruit mesocarp. *Fitoterapia* 2000; 71:190-92.
20. Parra A L, Bucla LI. Comparative study of the assay of *Artemia salina* L. and the estimation of the medium lethal dose (LD50 value) in mice, to determine oral acute toxicity of plant extracts. *Phytomedicine* 2001; 8(5): 395-400.
21. Ahmad MS, Mirza B. Synthesis, chemical characterization and biological screening for cytotoxicity and antitumor activity of Organotin(IV) derivatives of 3,4-Methylenedioxy 6-nitrophenylpropenoic acid. *Molecules* 2007; 12: 2348-63.

22. Genupur A, Jesu JLR, Srinivasan N, et al. Synthesis and cytotoxicity of novel isomeric C-seco limonoids. *Eur J Med Chem* 2006; 41: 997-1002.
23. Datki Z, Juhasz A, Galfi M, et al. Method for measuring neurotoxicity of aggregating polypeptides with the MTT assay on differentiated neuroblastoma cells. *Brain Res Bull* 2003; 62: 223-29.
24. Ribeiro-Dias F, Barbuto J, Tsujita M, Jancar S. Discrimination between NK and LAK cytotoxic activities of murine spleen cells by MTT assay: differential inhibition by PGE and EDTA. *J Immunol Meth* 2000; 241: 121-29.
25. Wanyoike G, Chhabra S, Langat-Thoruwa C, Omar S. Brine shrimp toxicity and antiplasmodial activity of five Kenyan medicinal plants. *J Ethnopharmacol* 2004; 90: 129-33.
26. Mackeen M, Ali A , Lajis N, et al. Antimicrobial, antioxidant, antitumour-promoting andcytotoxic activities of different plant part extracts of *Garcinia atroviridis* Griff. ex T. Anders. *J Ethnopharmacol* 2000; 72: 395-402.
27. Inoue M, Ohtani K, Kasai R, et al. Cytotoxic 16-b-[(D-xylopyranosyl) oxy] oxohexadecanyl triterpene glycosides from a Malagasy plant, *Physena sessiliflora*, *Phytochemistry* 2009; 70: 1195-1202.
28. Padmaja R, Arun P, Prashanth D, et al. Brine shrimp lethality bioassay of selected Indian medicinal plants. *Fitoterapia* 2002; 73: 508-510.
29. Pisutthanan S, Plianbangchang P, Pisutthanan N, et al. Brine Shrimp Lethality Activity of Thai Medicinal Plants in the Family Meliaceae. *Naresuan Uni J* 2004; 12(2):13-18.
30. Krishnaraju AV, Tsay H S. Biological Screening of medicinal plants collected from easternghats of India using *Artemia Salina. Int J applied sci & Eng* 2006; 4(2): 115-25.
31. Alim, MA, Khan AA, Rahman BM, et al. *In vitro* Antimicrobial properties and cytotoxic activities of chromium complexes. *Res J Agri & Biol Sci* 2007; 3(6): 599-604.
32. Carballo J, Hernandez-Inda Z, Perez P, et al. A comparison between two brine shrimp assays to detect in vitro cytotoxicity in marine natural products. *BMC Biotechnol* 2002; 2: 17.

33. Lee S, Min B, Kho Y. Brine shrimp lethality of the compounds from Phryma leptostachya L. *Arch Pharm Res* 2002; 25(5): 625-54.
34. Metcalf J, Lindsay J, Beattie K, et al. Toxicity of cylindrospermopsin to the brine shrimp *Artemia salina*: comparision with protein synthesis inhibitors and microcystins. *Toxican* 2002; 40: 1115-20.
35. Srivastava K, Mishra K. Cytogenetic effects of commercially formulated atrazine on the somatic cells of *Allium cepa* and *Vicia faba*. *Pest Biochem and Physiol* 2009; 93: 8-12.
36. Marcano L, Carruyo I, Campo D, et al. Cytotoxicity and mode of action of maleic hydrazide in root tips of Allium cepa L. *Environ Res* 2004; 94: 221-26.
37. Imbert D, Cullander C. Buccal mucosa in vitro experiments I. Confocal imaging of vital staining and MTT assays for the determination of tissue viability. *J Control Rel* 1999; 58: 39-50.
38. Elgie AW, Sargent JM, Taylor CG, et al. An *in vitro* study of blast cell metabolism in acute myeloid leukaemia using the MTT assay, *Leuk Res* 1996; 20(5): 407-13.
39. Kaspers G, Veerman A, Pieters R, et al. Drug combination testing in acute lymphoblastic leukemia using the MTT assay. *Leuk Res*1995; 19 (3): 175-81.