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R E S E A R C H A R T I C L E

Evaluation of *in-vitro* Antitumor Property of Water Weeds (*Eichhorniacrassipes* and *Pistiastratiotes*) by MTT Assay

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A B S T R A C T

Abstract: The water weeds *Eichhorniacrassipes* and *Pistiastratiotes* were selected to investigate the antitumor property *in-vitro* by MTT Assay. The IC50 (IC-Inhibitory Concentration) was calculated for all the fractions of the selected aquatic plants isolated through column chromatography. The fractions were subjected for HPLC analysis. At higher concentration of drug (500mg) less viable cells were found. Pistia have shown better cytotoxicity than Eichhornia when subjected to B16F10 cells, the % of viable cells scored at 500mg of aqueous fractions was 26 and 35 respectively. The IC50 of HPLC eluted fractions of Eichhornia & Pistia was found between 200 and 500mg/ml but the crude extracts of these water weeds did not respond similarly. The crude and alcoholic fractions of *Eichhorniacrassipes* and aqueous and methanolic fractions of *Pistiastratiotes* proved as potential tumor inhibitor.

Keywords: MTT, cytotoxicity, antitumor, *in-vitro*.

A R T I C L E I N F O

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CONTENTS

1. Introduction

Several plants containing flavonoid derivatives have found application as disease preventive and therapeutic agents in traditional medicine in Asia for thousands of years. The much lower risk of colon, prostate and breast cancers in Asians who consume more vegetables and fruits may indicate that flavonoid components act as natural chemo

preventive and anticancer agents. Experimental animal studies have indicated that certain dietary flavonoids possess antitumor activity (Cithan Kanadaswami *et al.*, 2005). The documented biological effects of flavonoids include anti-inflammatory, anti-allergic, antimicrobial, hepatoprotective, antiviral, antithrombotic, cardioprotective, capillary strengthening, antidiabetic, anticarcinogenic and antineoplastic effects, among others (Harborne JB & Williams CA, 2000; Middleton E Jr *et al.*, 2000; Kandaswami C & Middleton E Jr, 1994; Middleton E Jr & Kandaswami C, 1992; Middleton E Jr & Kandaswami C, 1994; Castillo MH *et al.*, 1989; Huang YT *et al.*, 1999; Kappagoda CT, 2005). Certain plant compounds, such as isoflavonoids, flavonoids and lignans, are receiving much attention as alleged cancer protective agents in populations with low incidences of breast and prostate cancer (Adlercreutz H *et al.*,, 1991).

Several flavonoids not only inhibit the growth of tumor cells (Middleton E Jr *et al.*, 2000; Huang YT *et al.*, 1999; Soulinna EM *et al.*, 1975; Scambia G *et al.*, 1990; Kandaswami C *et al.*, 1991) but also induce cell differentiation (Constantinou A *et al.*, 1990). Earlier reports indicate that some flavonoids also show potent antitumor activity *in-vivo* (Edwards JM *et al.*, 1979; Molnar J *et al.*, 1981). MTT assay was performed to evaluate the antitumor properties of the water weeds. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) assay is based on the conversion of MTT into formazan crystals by viable cells which determines mitochondrial activity.

Generally the total mitochondrial activity is related to the number of viable cells, therefore this assay is used to measure the *in-vitro* cytotoxic effects of drugs on cell lines or primary patient cells (Van Meerloo J*et al.*, 2011).The basis of this assay is the reduction of the soluble yellow MTT tetrazolium salt to a blue insoluble formazan product by mitochondrial succinic dehydrogenase (Mosmann T, 1983). This assay has also been used to detect cytotoxic lymphokines produced by human lymphocytes (Green *et al.*, 1984) and to measure cell activation (Gerlier and Thomasett, 1986).

2. Materials and Method

In-vitro tumor cell viability (IC50) was performed through MTT cytotoxicity assay.B16F10 cells were cultured *in-vitro* and subjected for monolayer growth. The tumor cell B16F10 melanoma cytotoxicity and tumor inhibitory property was scored by MTT assay. This assay was carried out as per the standard procedure described by (Fischer *et al*., 2003; Dutta *et al*., 2008) with slight modifications. Different concentrations from 25mg/ml–500mg/ml of *Eichhorniacrassipes* crude extract, Eichhornia crystals, methanolic fraction and aqueous fraction were taken.The cells were grown in fresh culture medium supplemented with 10% FBS and 1% Penicillin-Streptomycin mixture. The growth inhibition behavior of samples were evaluated by 3-(4, 5-di-methylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide (MTT) cytotoxicity assay. The cells were seeded evenly into 96-well flat-bottomed tissue culture plate at $5X10^3$ cells/well concentration and incubated for 24hrs in a humidified atmosphere of 5% CO_2 at 37^{o}C and treated with the formulations. Samples were applied as freshly prepared solutions in dimethylsulphoxide (DMSO). After 24 hours, 20ml of 5mg/ml MTT solution in PBS (pH 7.4) was added to each well and the plate was incubated for 2 hours at 37^{o}C allowing viable cells to reduce the MTT into purple colored formazan crystals. The absorbance was measured at 570 nm with the help of an ELISA plate reader.

3. Result and Observation

The tumor cell B16F10 melanoma cytotoxicity was performed by MTT assay. According to the Table 4 to 9, the methanolic fraction of Eichhornia have shown better cytotoxicity than Pistia when subjected to B16F10 cells. The percentage of viable cells scored at 500mg was found to be better in all the isolates where the viability of cells found to be less than 40%. Gallic acid, Catechin, Epicatechin of methanolic fraction of *Eichhorniacrassipes* have shown 28%, 33%, 35% of viable cells respectively whereas Gallic acid, Rutin of aqueous fraction of *Eichhorniacrassipes* have shown 36% and 38% viable cells respectively at 500mg/ml dose. Rutin of crude extract of *Eichhorniacrassipes* have shown 37% of viable cells whereas Rutin1 of aqueous fraction of *Eichhorniacrassipes* and Gallic acid of methanolic fraction of Pistia have shown 38% and 33% viable cells respectively.

Epicatechingallate, Ellagic acid, Rutin2, Quercetin, Kaempferol and Apigenin (Cunn, Kevin, 2000) have shown less than 40% viable cells in *Eichhorniacrassipes* and Pistia. Apigenin, unknown of crude extract of *Eichhorniacrassipes*, unknown 1, unknown 2 of aqueous extract of *Eichhorniacrassipes*, unknown and 1-O-Galloyl-B-D-Glucose of aqueous fraction of Pistia and unknown of methanolic fraction of Pistia could not able to present 50% of tumor cell death even at 500 mg/ml of dose. The IC 50 of HPLC eluted fractions of Eichhornia & Pistia have found between 200 and 500 mg/ml but the crude extracts of these water weeds could not able to present same response (Table 1 and 2). For Eichhornia crystal &methanolic fractions, the IC 50 was at 400 mg/ml where as the Pistia acetone fraction have not shown any 50% cell death even at 500 mg/ml. However the Methanolic and aqueous fractions have shown IC 50 at 400 mg/ml.

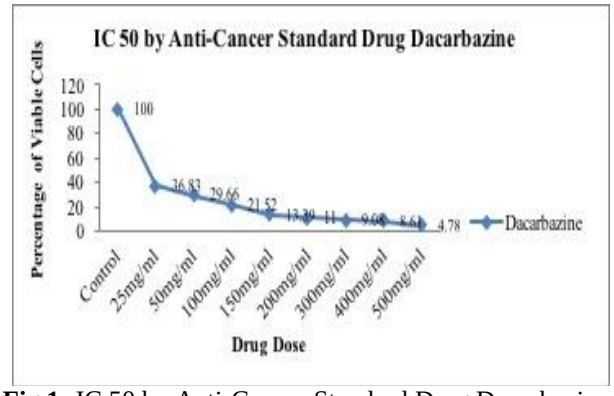

Fig.1: IC 50 by Anti-Cancer Standard Drug Dacarbazine

Table 1: MTT Assay of Eichhornia against B16F10 Cells

Dose	E. Crude (OD)	Viable Cells (%)	E. Crystal (OD)	Viable Cells (%)	EF1 (OD)	Viable Cells (%)	EF2 (OD)	Viable Cells (%)
Control	2.09	100	-	-	-	-	-	-
25mg/ml	1.99	95.2	2	95.69	1.91	91.38	1.8	86.12
50mg/ml	1.62	77.5	1.89	90.4	1.9	90.9	1.69	80.86
100mg/ml	1.43	68.42	1.67	79.9	1.79	88.64	1.63	77.99
150mg/ml	1.37	65.55	1.63	77.9	1.7	81.33	1.52	72.77
200mg/ml	1.07	51.96	1.44	68.89	1.5	71.77	1.41	67.46
300mg/ml	0.97	46.41	1.12	53.38	1.33	63.63	1.02	48.8
400mg/ml	0.73	34.9	0.82	38.27	0.97	46.41	0.82	39.23
500mg/ml	0.69	33.06	0.77	36.84	0.83	39.71	0.74	35.4

E. crystal- *Eichhorniacrassipes* crystals, EF1- *Eichhorniacrassipes*m ethanolic fraction, EF2- *Eichhorniacrassipes* aqueous fraction,PF1- *Pistiastratiotes* acetone fraction, PF2- *Pistiastratiotes*m ethanolic fraction, PF3- *Pistiastratiotes* aqueous fraction, *B16F10- (200000 cells/well)

Table 2: MTT Assay of Pistia against B16F10 Cells

Dose	Pistia Crude (OD)	Viable Cells (%)	PF1 (OD)	Viable Cells (%)	PF2 (OD)	Viable Cells (%)	PF3 (OD)	Viable Cells (%)
Control	2.09	100	-	-	-	-	-	-
25mg/ml	2.03	97.1	2.1	100	1.9	90.9	1.97	94.25
50mg/ml	1.99	95.2	1.93	92.33	1.82	87.08	1.79	85.64
100mg/ml	1.81	86.6	1.91	91.37	1.77	84.68	1.57	75.11
150mg/ml	1.82	87.08	1.82	87.06	1.66	79.42	1.32	63.15
200mg/ml	1.7	81.33	1.76	83.71	1.62	77.51	1.27	60.76
300mg/ml	1.56	74.64	1.6	76.51	1.43	68.42	1.07	51.96
400mg/ml	1.11	53.1	1.42	67.91	1.01	48.32	0.88	42.1
500mg/ml	0.82	39.22	1.37	65.88	0.83	39.71	0.56	26.79

***B16F10- (200000 cells/well)**

Table 3: MTT Assay of Standard Anticancer Drug Dacarbazine against B16F10 Cells

Dacarbazine Dose	Dacarbazine (OD)	Viable Cells (%)
0.1 µl	0.77	36.83
0.2 µl	0.62	29.66
0.3 µl	0.45	21.52
0.4 µl	0.28	13.39
0.5 µl	0.23	11
0.6 µl	0.19	9.08
0.7 µl	0.18	8.61
0.8 µl	0.1	4.78

Dacarbazine Dose- 250mg/m^2,*B16F10- (200000 cells/well)

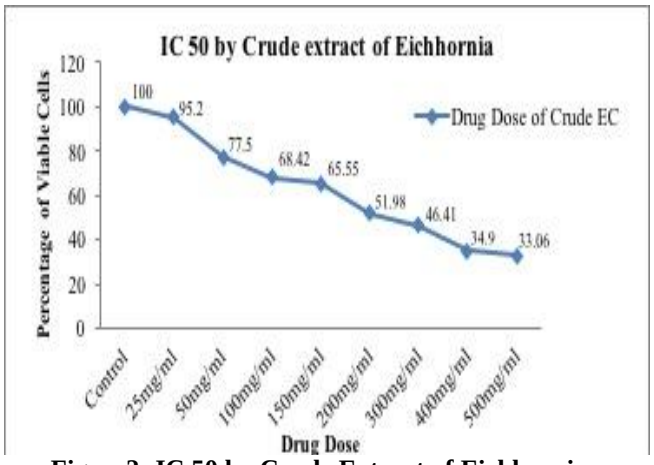

Figure2: IC 50 by Crude Extract of Eichhornia

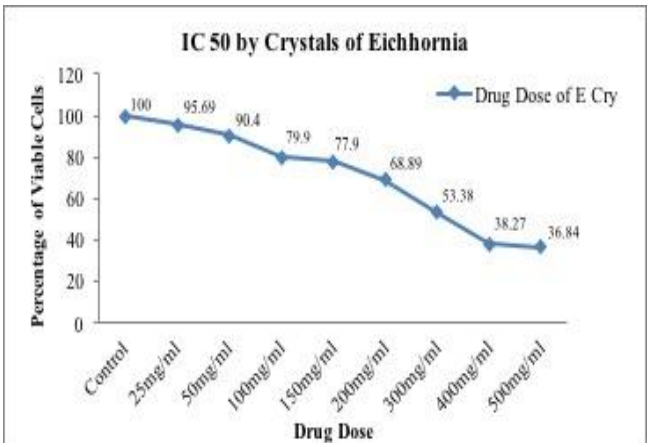

Figure3: IC 50 by Crystals of Eichhornia

Table 4: MTT Cellular Toxicity Assay of Methanolic Extract of Eichhorniacrassipes isolated through HPLC against B16F10 Cells

Sample Name	Compound Isolated	Dose Groups: A1, A2 – 200mg/ml B1, B2 – 500mg/ml	OD	Mean	% Viability
Methanolic fraction of ***Eichhorniacrassipes***	Gallic acid	A1	0.704	A-0.661	A-31.6%
		A2	0.619		
		B1	0.632	B-0.599	B-28.6%
		B2	0.566		
	Catechin	A1	0.847	A-0.736	A-35.2%
		A2	0.625		
		B1	0.801	B-0.702	B-33.5%
		B2	0.603		
	Epicatechin	A1	0.604	A-0.755	A-36.1%
		A2	0.906		
		B1	0.884	B-0.745	B-35.6%
		B2	0.606		
	Epicatechingallate	A1	0.806	A-0.856	A-40.9%
		A2	0.907		
		B1	0.815	B-0.761	B-36.4%
		B2	0.708		
	Apigenin	A1	0.936	A-0.857	A-41%
		A2	0.778		
		B1	0.801	B-0.727	B-34.7%
		B2	0.653		

***B16F10- (100000 cells/well)**

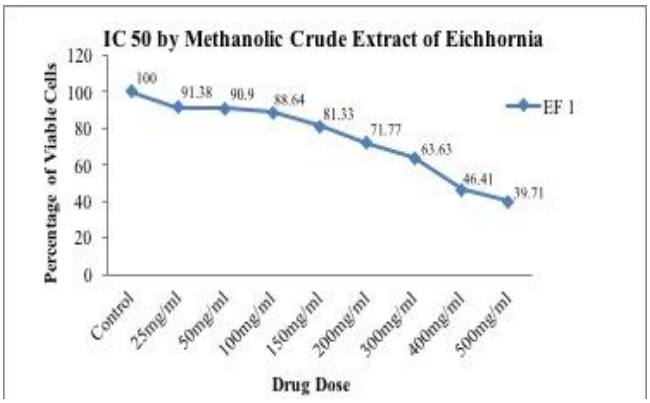

Figure 4: IC 50 by Methanolic Crude Extract of Eichhornia

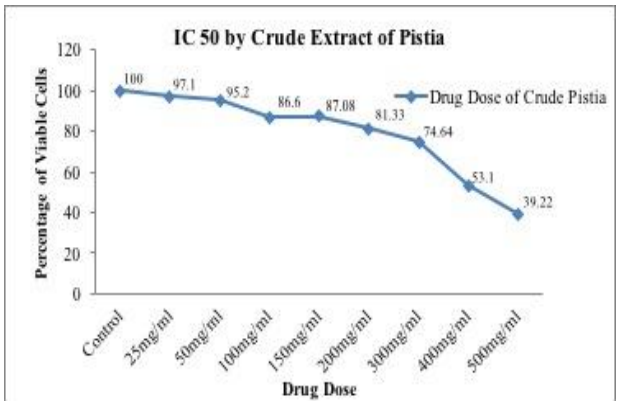

Figure 6: IC 50 by Crude Extract of Pistia

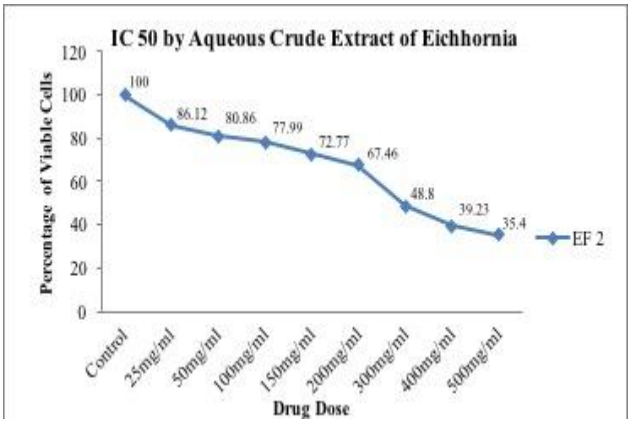

Figure5: IC 50 by Aqueous Crude Extract of Eichhornia

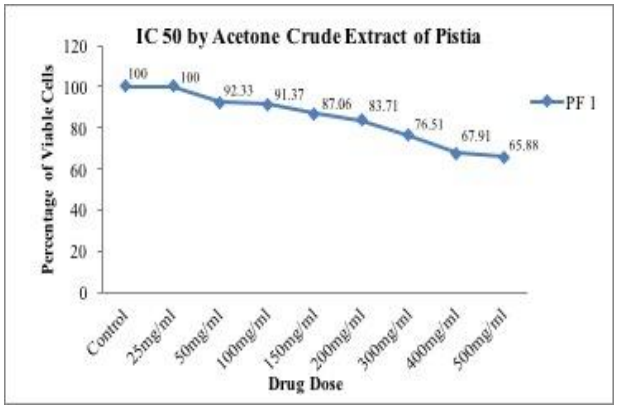

Figure7: IC 50 by Acetone Crude Extract of Pistia

Table 5: MTT Cellular Toxicity Assay of Aqueous Extract of *Eichhorniacrassipes* isolated through HPLC against B16F10 Cells

Sample Name	Compound Isolated	Dose Groups:A1, A2 – 200mg/ml B1, B2 – 500mg/ml	OD	Mean	% Viability
Aqueous fraction of***Eichhorniacrassipes***	Gallic acid	A1	0.712	A-0.769	A-36.7%
		A2	0.826		
		B1	0.808	B-0.761	B-36.4%
		B2	0.714		
	Epigallocatechin	A1	0.778	A-0.857	A-41.0%
		A2	0.936		
		B1	0.866	B-0.849	B-40.6%
		B2	0.832		
	Protocatechuic Acid	A1	0.991	A-0.899	A-43.0%
		A2	0.808		
		B1	0.964	B-0.848	B-40.5%
		B2	0.732		
	Unknown 1	A1	1.305	A-1.134	A-54.2%
		A2	0.963		
		B1	1.234	B-1.109	B-53.0%
		B2	0.984		
	Unknown 2	A1	1.378	A-1.33	A-63.6%
		A2	1.282		
		B1	0.903	B-1.093	B-52.2%
		B2	1.283		
	Rutin 1	A1	0.809	A-0.813	A-38.8%
		A2	0.817		
		B1	0.874	B-0.801	B-38.3%
		B2	0.729		
	Rutin 2	A1	0.805	A-0.862	A-41.2%
		A2	0.92		
		B1	0.804	B-0.835	B-39.9%
		B2	0.867		
	Ellagic Acid	A1	0.929	A-0.904	A-43.2%
		A2	0.879		
		B1	0.861	B-0.92	B-44.0%
		B2	0.979		
	Vallinic Acid	A1	1.145	A-1.042	A-49.8%
		A2	0.94		
		B1	0.907	B-1.006	B-48.1%
		B2	1.105		
	Kaempferol	A1	0.984	A-0.942	A-45.0%
		A2	0.901		
		B1	0.892	B-0.854	B-40.8%
		B2	0.817		
	Myricetin	A1	1.01	A-0.96	A-45.9%
		A2	0.91		
		B1	0.949	B-0.900	B-43.0%
		B2	0.852		
	Quercetin	A1	0.769	A-0.866	A-41.4%
		A2	0.963		
		B1	0.803	B-1.003	B-47.9%
		B2	1.204		
	Naringinin	A1	0.905	A-1.006	A-48.1%
		A2	1.107		
		B1	0.869	B-0.881	B-42.1%
		B2	0.894		

Table No. 6: MTT Cellular Toxicity Assay of Crude Extract of *Eichhorniacrassipes* isolated through HPLC Analysis against B16F10 Cells

Sample No.	Compound Isolated	Dose Groups	OD	Mean	% Viability
Crude Extract of***Eichhorniacrassipes***	Rutin	A1-200mg/ml A2-200mg/ml B1-500mg/ml B2-500mg/ml	0.742 0.829 0.879 0.685	A-0.785 B-0.782	A-37.5% B-37.4%
	Ellagic Acid	A1-200mg/ml A2-200mg/ml B1-500mg/ml B2-500mg/ml	0.787 0.910 0.836 0.834	A-0.848 B-0.835	A-40.5% B- 39.9%
	Vallinic Acid	A1-200mg/ml A2-200mg/ml B1-500mg/ml B2-500mg/ml	0.912 1.098 1.039 0.932	A-1.005 B-0.985	A-48.0% B-47.1%
	Kaempferol	A1-200mg/ml A2-200mg/ml B1-500mg/ml B2-500mg/ml	0.945 0.830 0.852 0.893	A-0.887 B-0.872	A-42.4% B-41.7%
	Unknown	A1-200mg/ml A2-200mg/ml B1-500mg/ml B2-500mg/ml	1.989 1.127 0.901 1.278	A-1.558 B-1.089	A-61.2% B-52.1%
	Myricetin	A1-200mg/ml A2-200mg/ml B1-500mg/ml B2-500mg/ml	0.965 0.950 0.829 1.079	A-0.957 B-0.954	A-45.7% B-45.6%
	Quercetin	A1-200mg/ml A2-200mg/ml B1-500mg/ml B2-500mg/ml	0.826 0.860 0.849 0.828	A-0.843 B-0.838	A-40.3% B-40.0%
	Astragalin	A1-200mg/ml A2-200mg/ml B1-500mg/ml B2-500mg/ml	0.985 0.798 0.786 0.961	A-0.891 B-0.873	A-42.6% B-41.7%
	Caffeic Acid	A1-200mg/ml A2-200mg/ml B1-500mg/ml B2-500mg/ml	0.908 1.118 1.109 0.978	A-1.013 B-1.043	A-48.4% B-49.9%
	Apigenin	A1-200mg/ml A2-200mg/ml B1-500mg/ml B2-500mg/ml	0.945 1.240 1.087 1.010	A-52.2% B-1.048	A-52.2% B-50.1%
	Naringinin	A1-200mg/ml A2-200mg/ml B1-500mg/ml B2-500mg/ml	0.938 1.028 0.997 0.982	A-0.983 B-0.989	A-47.0% B-47.3%

***B16F10- (100000 cells/well)**

Table No.7: MTT Cellular Toxicity Assay of Acetone Extract of *Pistiastratiotes* isolated through HPLC against B16F10 Cells

Sample No.	Compound Isolated	Dose Groups	OD	Mean	%Viability
Acetone fraction of***Pistiastratiotes***	Gallic acid	A1-200mg/ml	0.837	A-0.864	A-41.3%
		A2-200mg/ml	0.892		
		B1-500mg/ml	0.874	B-0.801	B-38.3%
		B2-500mg/ml	0.729		
	Rutin	A1-200mg/ml	0.961	A-0.97	A-46.4%
		A2-200mg/ml	0.979		
		B1-500mg/ml	0.841	B-0.885	B-42.3%
		B2-500mg/ml	0.929		
	Vallinic Acid	A1-200mg/ml	1.034	A-1.01	A-48.3%
		A2-200mg/ml	0.986		
		B1-500mg/ml	1.019	B-0.984	B-47.0%
		B2-500mg/ml	0.95		

***B16F10- (100000 cells/well)**

Table No. 8: MTT Cellular Toxicity Assay of Aqueous Extract of *Pistiastratiotes* isolated through HPLC against B16F10 Cells

Sample No.	Compound Isolated	Dose Groups	OD	Mean	%Viability
Aqueous fraction of***Pistiastratiotes***	Gallic acid	A1-200mg/ml	0.817	A-0.839	A-40.1%
		A2-200mg/ml	0.862		
		B1-500mg/ml	0.837	B-0.838	B-40.0%
		B2-500mg/ml	0.839		
	1-O-Galloyl – β-D-Glucose	A1-200mg/ml	1.55	A-1.233	A-58.9%
		A2-200mg/ml	0.916		
		B1-500mg/ml	0.982	B-1.022	B-48.8%
		B2-500mg/ml	1.603		
	Rutin	A1-200mg/ml	0.893	A-0.869	A-41.5%
		A2-200mg/ml	0.845		
		B1-500mg/ml	0.902	B-0.863	B-41.2%
		B2-500mg/ml	0.825		
	Catechin	A1-200mg/ml	1.082	A-0.945	A-45.2%
		A2-200mg/ml	0.808		
		B1-500mg/ml	0.983	B-0.906	B-43.3%
		B2-500mg/ml	0.829		
	Epicatechin	A1-200mg/ml	0.92	A-0.959	A-45.8%
		A2-200mg/ml	0.999		
		B1-500mg/ml	0.94	B-0.956	B-45.7%
		B2-500mg/ml	0.972		
	Epicatechingallate	A1-200mg/ml	0.934	A-0.990	A-47.3%
		A2-200mg/ml	1.047		
		B1-500mg/ml	0.854	B-0.981	B-46.9%
		B2-500mg/ml	1.109		
	Unknown	A1-200mg/ml	1.923	A-1.454	A-69.5%
		A2-200mg/ml	0.986		
		B1-500mg/ml	1.625	B-1.15	B-55.0%
		B2-500mg/ml	0.675		

***B16F10- (100000 cells/well)**

Table No. 9: MTT Cellular Toxicity Assay of Methanolic Extract of *Pistiastratiotes* isolated through HPLC against B16F10 Cells

Sample No.	Compound Isolated	Dose Groups	OD	Mean	%Viability
Methanolic fraction of***Pistiastratiotes***	Gallic acid	A1-200mg/ml	0.894	A-0.849	A-40.6%
		A2-200mg/ml	0.805		
		B1-500mg/ml	0.194	B-0.703	B-33.6%
		B2-500mg/ml	1.212		
	Rutin	A1-200mg/ml	0.932	A-0.894	A-42.7%
		A2-200mg/ml	0.856		
		B1-500mg/ml	0.804	B-0.890	B-42.5%
		B2-500mg/ml	0.977		
	Catechin	A1-200mg/ml	0.966	A-0.933	A-44.6%
		A2-200mg/ml	0.9		
		B1-500mg/ml	0.983	B-0.906	B-43.3%
		B2-500mg/ml	0.829		
	Unknown	A1-200mg/ml	1.076	A-1.091	A-52.2%
		A2-200mg/ml	1.106		
		B1-500mg/ml	1.101	B-1.038	B-49.6%
		B2-500mg/ml	0.976		
	Kaempferol	A1-200mg/ml	0.92	A-0.959	A-45.8%
		A2-200mg/ml	0.999		
		B1-500mg/ml	0.914	B-0.935	B-44.7%
		B2-500mg/ml	0.956		

***B16F10- (100000 cells/well)**

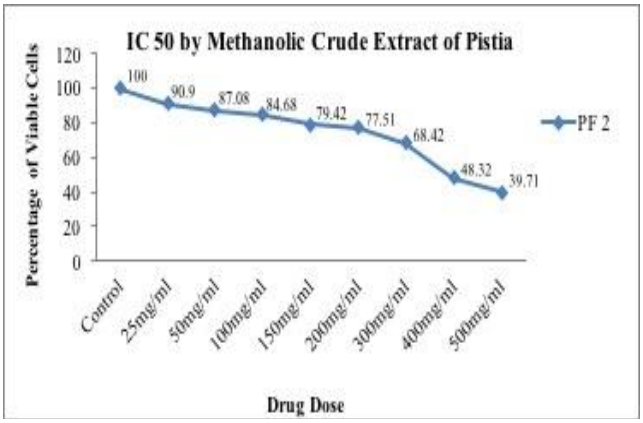

Figure 8: IC 50 by Methanolic Crude Extract of Pistia

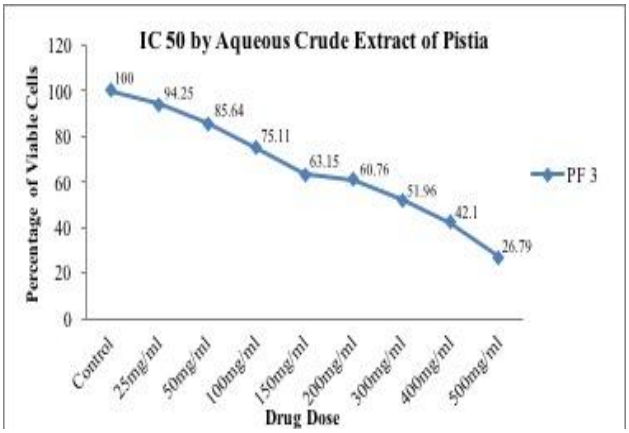

Figure 9: IC 50 by Aqueous Crude Extract of Pistia

The Optical Density was screened by ELISA Plate Reader ELX-808 iu/AVL Biomedical.

Control without any drug

Test Group

Dacarbazine

Figure 10: MTT Assay for Crude Extract of Eichhornia and Pistia compared to Dacarbazine

4. Discussion and Conclusion

It was found that Eichhornia crude inhibited 46.4% of the cells and aqueous fractions inhibited 48.8% of the cell. But Eichhornia crystals and methanolic fraction inhibited 38.27% and 46.41% of tumor cells. On the other hand, Pistiamethanolic fraction and aqueous fraction were able to inhibit 50% of cancer cells at 400mg/ml dose whereas Pistia crude able to inhibit at 500mg/ml. Acetone fraction has not shown 50% cell death even 500mg/ml. The IC 50 of *Eichhorniacrassipes* and Pistia against B16F10 cells was compared with dacarbazine. At very low concentration 25mg/ml of dacarbazine was able to inhibit 36.83% of the total cells. Gallic acid, Catechin, Epicatechin, Epicatechingallate of methanolic fraction of Eichhornia have shown best tumor cell inhibiting property than methanolic fraction of Pistia. Gallic acid and Rutin 1 of aqueous fraction of Eichhornia have shown better result than aqueous fraction of Pistia.

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Conflict of Interest: The authors declare that there are no conflicts of interest.

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