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Cytotoxic Evaluation of Microwave Assisted Ethanolic Extract of *Annona Muricata* Leaves

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ABSTRACT

Cancer is the very dangerous cause of death in the world. Day by day the scientists are struggling to get a permanent solution. The soursop (*Annona muricata*) is a medicinal plant which is used traditionally as antitumor agent. The cytotoxic effects from extracts of leaves of soursop in cancer cells L929 fibroblast, HCT colon cancer cells, MCF7, HeLa, SIHA cell lines were done under this study. The extraction was carried out by microwave assisted extraction method using ethanol and fractionation by column chromatography. The various solvents were Petroleum ether, Petroleum ether: chloroform (4:3, 3:2, 2:3 and 1:4), Chloroform, Chloroform: Ethanol (4:3, 3:2, 2:3 and 1:4). Such ten fractions were collected. Cytotoxic test was done by the method of MTT assay by using the extract at different concentrations and the IC50 value was calculated ie values that produce inhibitory concentrations of cancer cells by 50%. At 50µg/ml the same assay was repeated by using ten different fractions. The results showed that the ethanolic extracts of leaves of soursop has a cytotoxic activity against L929 cell lines, HCT colon cell lines, MCF 7, SIHA and HeLa with IC50 values of 68.5, 187.5, 112.6, 23.4 and 24.9 µg/ml respectively. Among the ten different fractions at 50µg/ml, the fraction AM3 was showed best cytotoxic activity. In case of AM3, IC50 value was found to be 16µg / ml. The fraction AM3 can be selected for further study.

INTRODUCTION

Cancer is a deadly disease where the cells divide faster. Cancer is caused by certain environmental factors [1]. The treatment is so difficult due to its wide and fast spread. Researchers are trying their level best to treat this disease. Since the number of patients and the number of deaths continued to increase, this must be accompanied by curative efforts. A vast variety of medicines were available but the side effects are also horrible.

Recently one of the plants has a greater attention in the society which is the leaves of the soursop (*Annona muricata Linn.*). It is using traditionally for cancer treatment [2]. *Annona muricata* is also known as corossol [3]. Literatures showing as the Annonaceae family is having a variety of pharmacological actions including the cell inhibitory action. *Annona montana* contains monotetrahydrofuranic acetogenins which have toxicity to liver cancer in Hep G2 cells [4]. The seeds of *Annona crassiflora* have high antioxidant activity [5]. *Annona squamosa* containing an immunotoxin it is used for the treatment of cancer [6]. Based on the study of chemotaxonomy of these plants they may contain similar components. The previous studies showed that the plant *Annona muricata* (soursop) is a potent anticancerous plant from the Annonaceae family.

MATERIALS AND METHODS

Plant material

The plant *Annona muricata* belonging to the family Annonaceae was collected from Neyyattinkara, Thiruvananthapuram District, Kerala. It was authentified by Dr. Jomy Augustine, Department of Botany, St. Thomas College, Pala, Kottayam, Kerala. A specimen with voucher no.1501 was kept in the department for further reference.

Microwave assisted extraction and fractionation

The leaves of *Annona muricata* were dried under shade and then powdered with a mechanical grinder. The powder was passed through sieve No. 42 and stored in an airtight container for further use. The dried material was extracted in ethanol by scientific microwave assisted extraction method [7]. The

extraction time was 5 minutes and the power used was 850W. About 30 ml of ethanol was used as the solvent. The extract was made solvent free by distillation process and the resulting semisolid mass was vacuum dried to yield a solid residue. The experiment was repeated to get 20g of the extract.

Fractionation was done through column chromatography started by using 20g of the extract. Slurry of the extract was prepared in ethanol and the extract was uniformly packed over dry silica gel (mesh size 230-400, 20g). Petroleum ether, Petroleum ether: chloroform (4:3, 3:2, 2:3 and 1:4), Chloroform, Chloroform: Ethanol (4:3, 3:2, 2:3 and 1:4) such different solvents in different ratios were used as the mobile phase. 98 small fractions were collected in small test tubes. TLC (silica gel F254) of all individual fractions was developed. It was then viewed under UV chamber. Based on the TLC results similar fractions were pooled. The fractions were dried in rotavapor under reduced pressure at a temperature of about 40±5º. Such 10 combined fractions were collected.

MTT assay

Preparation of stock solutions of test material

The soursop leaf extract (5mg) is weighed, followed by retrieval of DMSO to 5 ml (stock solution concentration of 1 mg / ml) and stored as stock solutions. It can be used for subsequent use in research. Cytotoxic concentration of extract to a test carried out by using the dilution medium. Tamoxifen concentrations obtained by dilution with medium. As a control solvent, used 2% DMSO (v/v), i.e. the highest concentration of DMSO in the test compound.

Cytotoxic test with soursop leaf extract on cell lines by MTT assay [8]

The cell lines were purchased from NCCS Pune was maintained in Dulbecco's modified eagles media (HIMEDIA) supplemented with 10% FBS (Invitrogen) and grown to confluency at 37°c in 5 % CO_2 (NBS, EPPENDORF, GERMANY) in a humidified atmosphere in a CO_2 incubator. The cells were trypsinized (500µl of 0.025% Trypsin in PBS/ 0.5mM EDTA solution (Himedia) for 2 minutes and transfer to T flasks in complete aseptic conditions. Extracts were added to grown cells at different concentrations from a stock of 10 mg/ml and incubated for 24 hours. The % difference in viability was determined by standard MTT assay after 24 hours of incubation. The wells containing polymer and cells were washed with 1x PBS and then added 50 µl of MTT solution to the culture (MTT - 5mg/ml dissolved in PBS). It was then incubated at 37^{0}C for 3 hours. MTT was removed by washing with 1x PBS and the formazan was eluted out with 200 µl of Isopropanol. Incubation was done at room temperature for 30 minutes until the cell got lysed and colour was obtained. The solution was transferred to centrifuge tubes and centrifuged at top speed for 2minutes to precipitate cell debris. Optical density was read at 540 nm using DMSO as blank using an ELISA reader (LISASCAN, Erba). Cytotoxicity test is a qualitative and quantitative tests to determine how cell death. Optical density value of each test compound can be seen in Table 1. Optical density of the control is calculated and the percentage viability can be calculated by using the following formula.

Percentage viability = Optical density of test /Optical density of control X 100

RESULTS

The extraction of *Annona muricata* leaves was done by microwave assisted extraction method. The extract obtained was subjected to the cytotoxic activity testing in different cell lines. The IC50 value was calculated and in case of L929 cell lines it was found to be 68.5µg/ml, in case of HCT Colon cell lines 187.5µg/ml, MCF7 cell lines 112.6µg/ml, SIHA 23.4µg/ml, and in case of HeLa 24.9µg/ml. The results are shown in the table 1.

The cell inhibitory action against SIHA was better than the action against other cell lines since the IC50 value was found to be 23.4µg/ml. At 50µg/ml the same study was done by using the different fractions of *Annona muricata* ethanol extract. The results are shown in the table 2. The fraction AM3 showed better action compared to others. The percentage inhibition against MCF7 was more and it was found to be 83.24 %. The IC_{50} value of fraction 3 against MCF7 was found to be 16 µg/ml. The results are shown in the table 3. The concentration vs percentage inhibition

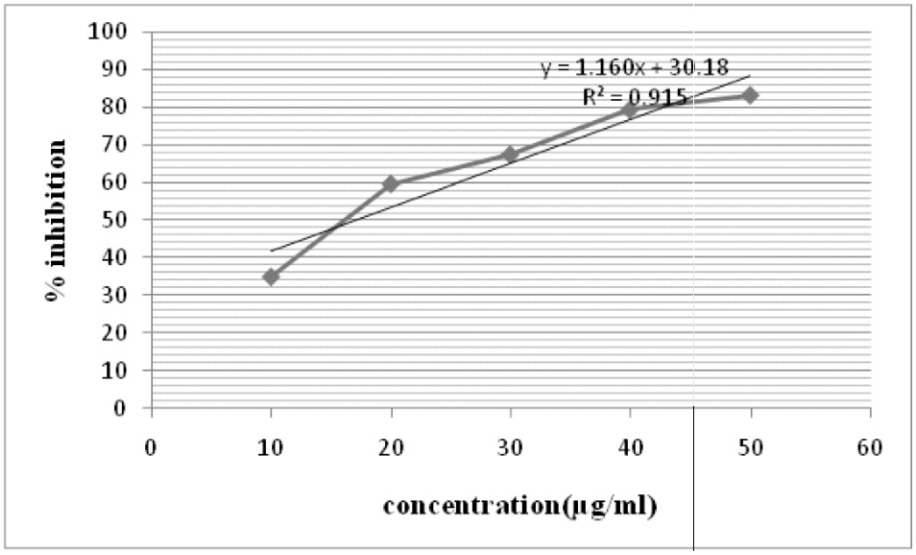

Fig 1: Concentration vs percentage inhibition graph of AM3

Table 1: The percentage inhibition and IC50 values of the *Annona muricata* leaf extract on different cell lines

Cell lines used	Concentration (µg/mL)	Average OD at 540 nm	Viability (%)	Retardation (%)	IC_{50} (µg/ml)
L929	1000	0.0411	7.19	92.81	68.5
	500	0.0512	8.95	91.05	
	250	0.1062	18.57	81.43	
	125	0.1877	32.82	67.18	
	62.5	0.2876	50.29	49.71	
	31.25	0.3187	55.73	44.27	
	15.6	0.3254	56.90	43.10	
HCT 15	1000	0.1012	17.69	82.30	187.5
	500	0.2012	35.18	64.82	
	250	0.2152	37.63	62.37	
	125	0.3412	59.66	40.34	
	62.5	0.3764	65.82	34.18	
	31.25	0.3812	66.66	33.34	
	15.6	0.4123	72.09	27.91	
MCF7	1000	0.1121	19.60	80.39	112.6
	500	0.1814	31.72	68.28	
	250	0.2200	38.47	61.53	
	125	0.2814	49.20	50.79	
	62.5	0.3231	56.49	43.50	
	31.25	0.3512	61.41	38.59	
	15.6	0.3621	63.32	36.68	
SIHA	1000	0.0812	14.19	85.80	23.4
	500	0.1011	17.68	82.32	
	250	0.1231	21.52	78.48	
	125	0.1611	28.17	71.83	
	62.5	0.1842	32.21	67.79	
	31.25	0.2234	39.06	60.94	
	15.6	0.2912	50.92	49.08	
HELA	1000	0.0941	16.45	83.55	24.9
	500	0.1280	22.38	77.62	
	250	0.1612	28.19	71.81	
	125	0.1914	33.47	66.53	
	62.5	0.2210	38.64	61.36	
	31.25	0.2614	45.71	54.29	
	15.6	0.2999	52.44	47.56	
Tamoxifen	50	0.253	0	100	13.38
	25	0.262	0.19	99.81	
	12.5	0.889	71.62	28.38	
	6.25	1.070	91.95	8.05	
	3.125	1.167	92.29	7.71	

Table 2: The percentage viability and percentage inhibition of different fractions of *Annona muricata* leaf extract on different cell lines

Different cell lines	Test Materials (10µg/ml)	Average OD at 540 nm	Viability (%)	Retardation (%)
L929	Fraction 1	0.4862	46.3	53.7
	Fraction 2	0.4630	44.09	55.91
	Fraction 3	**0.3508**	**33.40**	**66.6**
	Fraction 4	0.5322	50.68	49.32
	Fraction 5	0.9878	94.07	5.93
	Fraction 6	0.4768	45.40	54.6
	Fraction 7	0.9075	86.42	13.58
	Fraction 8	0.9923	94.50	5.50
	Fraction 9	0.9520	90.66	9.34
	Fraction 10	0.7624	72.60	27.4
HCT COLON 15	Fraction 1	0.4110	39.14	60.86
	Fraction 2	0.3220	30.67	69.33
	Fraction 3	**0.2840**	**27.05**	**72.95**
	Fraction 4	0.4051	38.58	61.42
	Fraction 5	0.3840	36.57	63.43
	Fraction 6	0.3720	35.43	64.57
	Fraction 7	0.3210	30.57	69.43
	Fraction 8	0.4120	39.24	60.76
	Fraction 9	0.4321	41.15	58.85
	Fraction 10	0.5322	50.68	49.31
MCF7	Fraction 1	0.2940	28.00	72.00
	Fraction 2	0.3240	30.86	69.14
	Fraction 3	**0.1760**	**16.76**	**83.24**
	Fraction 4	0.4010	38.19	61.81
	Fraction 5	0.3120	29.71	70.29
	Fraction 6	0.3221	30.68	69.32
	Fraction 7	0.4020	38.29	61.71
	Fraction 8	0.2160	20.57	79.43
	Fraction 9	0.2840	27.05	72.95
	Fraction 10	0.3011	28.68	71.32
SIHA	Fraction 1	0.3160	30.09	69.90
	Fraction 2	0.3171	30.20	69.80
	Fraction 3	**0.1860**	**17.71**	**82.29**
	Fraction 4	0.1960	18.67	81.33
	Fraction 5	0.3021	28.78	71.22
	Fraction 6	0.3160	30.09	69.90
	Fraction 7	0.3230	30.76	69.24
	Fraction 8	0.3420	32.57	67.43
	Fraction 9	0.2872	27.35	72.65
	Fraction 10	0.2960	28.19	71.81
HeLa	Fraction 1	0.3220	30.67	69.33
	Fraction 2	0.4120	39.24	60.76
	Fraction 3	**0.1841**	**17.53**	**82.47**
	Fraction 4	0.2940	28.00	72.00
	Fraction 5	0.3120	29.71	70.29
	Fraction 6	0.3016	28.72	71.28
	Fraction 7	0.3114	29.66	70.34
	Fraction 8	0.4116	39.20	60.80
	Fraction 9	0.4121	39.25	60.75
	Fraction 10	0.3248	30.93	69.07

Table 3: Calculation of IC50 value in case of AM3 against MCF7 cell lines

Concentration (µg/ml)	Average OD at 540nm	% Viability	% Inhibition	IC50 value (µg/ml)
50	0.1760	16.76	83.24	
40	0.2160	20.57	79.43	
30	0.3412	32.49	67.50	16
20	0.4227	40.26	59.74	
10	0.6818	64.93	35.07	

of *Annona muricata* leaf fraction 3 against MCF7 is depicted in the fig.1.

DISCUSSION

Therapeutically desired herboconstituents can be separated by the method of extraction. Extraction means the solubilisation of secondary metabolites from the matrix. By the use of efficient solvents the target compounds can be separated. A number of procedures can be adopted for the extraction. In the present study adopted the microwave assisted extraction. Due to the microwave energy, the moisture content present in the cell get heated and the moisture get evaporated and the active constituents are coming out to the surrounding solvents.

Annona muricata leaves extract is having wide variety of activities including anticancer activity. Due to this reason a detailed cancer research is ongoing on this plant. The extracts are reported to display selective cell inhibition in various cell lines. The ethanol extract can trigger cell death and the percentage of inhibition increases on increasing concentration. L929, HCT colon cell lines, MCF7, HeLa, SIHA were used in this study.

The results from the study showed that the extract can inhibit the cell growth to a certain extent. The results also highlights that the cell inhibition against SIHA was greater and the extract can be used for the detailed study by using this cell lines. Among the fractions the fraction 3 since it is showing better activity against the MCF 7 cell lines since the highest activity is exhibited by the lowest IC50 value. That particular fraction need a detailed study to find out the exact active constituent responsible for the cell inhibitory action.

This study indicates that *Annona muricata* have its own valuable type of chemicals that contribute the effects. Most of the studies are by using its extracts, but the present study also focuses on its fractions separated from the extract. Since the plant is exhibiting extraordinary properties, the plant's importance is increasing day by day.

CONCLUSION

Annona muricata is a traditional medicinal plant having wide variety of pharmacological activities. In the present study the ethanol extract of leaves of soursop (*Annona muricata*) was prepared by microwave assisted extraction method. Microwave assisted extraction helps to reduce the extraction time and solvent volume consumed was also very less. The extract was subjected to cytotoxic activity on different cell lines. Absorbance data obtained, is used to calculate IC_{50} values. The smaller value of IC_{50} has the greater potential cytotoxic against cell lines. Among the different cell lines used, the cytotoxic activity against SIHA cell lines was greater than the other cell lines. But the different fractions of the ethanol extract while subjected to the same study, the fraction 3 has the best potency of cytotoxicity against MCF7 cell lines. The fraction that has a better cytotoxic potency against MCF7, was selected for further study.

Conflict of interest

Authors do not have any conflict of interest

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