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INVITRO AND INVIVO ANTICANCER ACTIVITY OF METHANOL EXTRACT OF NYMPHOIDES KRISHNAKESARA VAR.BISPINOSA

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ABSTRACT

Worldwide cancer is the major cause of death. Millions of people died due to different types of cancer such as lung cancer, liver cancer, breast cancer, stomach cancer, blood cancer etc. Couples of therapies like chemotherapy, radiation, surgery and targeted therapy are available with some detrimental effect. Natural phytochemicals are prominent strategy for prevention, treating, and curing cancer. There are many plants (phytochemicals) possess anti-cancer activity that exhibits additive or synergistic activity. Generally these phytochemicals treat cancer by different mechanisms like augmenting apoptosis, cell cycle arrest, targeting to some specific cancer inducing proteins, increasing cytotoxicity etc. In this study a methanol extractfrom whole plant *Nymphoides krishnakesara Var. Bispinosa* male and female plantswere evaluated for in vitro anticancer activity by MTT cell viability assay and in vivo anticancer activity by DAL (Dalton's Ascites Lymphoma) Induced Anticancer Study.The viability of cells were evaluated byMTT assay method.These studies indicates positive anticancer activity of the extractNymphoides krishnakesara Var. Bispinosa male and female (whole plant) by invitro as well as in vivo study.

Key Words: MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), DAL (Dalton's Ascites Lymphoma), synergistic, anticancer.

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INTRODUCTION

Nymphoides krishnakesara variety bispinosa is newly discovered aquatic submerged plant found and collected from the ponds in the laterite plateau of the Malabar Coast, Kerala, India.

N. macrospermum N. aurantiaca N. Sivarajanii N. peltata N. Hydrophylla N. indica N. parvifolia are the species of Nymphoides (Menyanthaceae) and are found to be used to treat anemia, epilepsy, jaundice, fever etc(1).

Cancer is not a single disease. It is a group of more than 200 different disease. Cancer can be generally described as an uncontrolled growth and spread of abnormal cells in the body. The treatment used depends on the type of cancer, like surgery, chemotherapy, radiation therapy.

MATERIAL AND METHODS

Plant material

The whole plants of Nymphoides Krishnakesara (both male and female)-Menyanthaceae collected from the pond of Kasaragod. The plant materials were taxonomically identified by the botanist, Mr. Biju, Assistant professor, Department of Botany, Government College, Kasaragod.

Extraction procedure

Plants were washed well and dried under shade for about 08 days, powdered with mechanical grinder and stored in an air tight container. Extraction of the dried powder of the *Nymphoides Krishnakesara*, was carried out by successive solvent extraction using solvents of increasing polarity viz. petroleum ether, benzene, chloroform, ethyl acetate, acetone and methanol. Around 25 g of dried powder was weighed, moistened with the respective solvent and packed in the soxhlet extractor and was then extracted with 500 ml each of the petroleum ether, benzene, chloroform, ethyl acetate, acetone and methanol. After each extraction, the same dried marc was used for the subsequent extraction. Each extract was then filtered, the solvent distilled off and finally the dried extract was obtained. The percentage yield of each extracts was calculated. These extracts were used for preliminary phytochemical screening (1, 2).

Preliminary phytochemical screening (2-4)

The preliminary phytochemical screening of all the extracts was carried out by standard procedure. The results are shown in table. Methanol extract was used for the anticancer study based on the in vitro-in vivo study carried out.

ANTICANCER ACTIVITY

IN VITRO ANTICANCER STUDIES OF METHANOL EXTRACT OF *NYMPHOIDES KRISHNAKESARA* (5)

MTT cell viability assay

The cell culture suspension was washed with 1 x PBS and then added 200µlMTT solution to the culture flask (MTT 5 mg/volume dissolved in PBS, filtered through a 0.2µm filter before being use). Then incubated at 37ºC for 3 hours, removed all MTT solution, washed with 1 x PBS and added 300µl DMSO to each culture flask, incubated at room temperature for 30 minutes until all cells get lysed and homogenous colour was obtained. The solution was then transferred to centrifuge tube and centrifuged at top speed for 2 minutes to precipitate cell debris (Table-1). OD was measured at 540 nm using DMSO blank. Then the percentage viability was calculated.

Calculation

$$\% \text{ of cell viability} = \frac{\text{Mean absorbance of sample} \times 100}{\text{Mean absorbance of control}}$$

Table-1 Reagents and solutions for MTT assay

Reagent	Preparation
PBS-EDTA solution	Readymade mix was procured from Invitrogen, cat.no.11668027.
MTT ($5mg.ml^{-1}$)	5 mg of MTT is prepared in serum free medium
MTT lysis buffer	Isopropanol procured from Invitrogen, cat.no. I0398

IN VIVO PHARMACOLOGICAL STUDIES OF METHANOLIC EXTRACT OF NYMPHOIDES KRISHNAKESARA BISPINOSA (MALE & FEMALE) (6, 7).

DAL (Dalton's Ascites Lymphoma) Induced Anticancer Study of *Nymphoides krishnakesara*

Acute Toxicity Study

Drug material

Methanol extract of *Female Nymphoides krishnakesara* (MEFNK) and Methanol extract of *MaleNymphoides krishnakesara* (MEMNK)

Experimental animals

Healthy Wistar Albino rats of either sex, weighing about 150-200 gm were procured from animal house. The entire study was approved by the Institutional Animal Ethical Committee (IAEC) which is certified by the Committee for the Purpose of Control and Supervision of Experiments on Animals. The animals were kept in clean and dry polycarbonate cages and maintained in a well-ventilated animal house with 12 hours light – 12 hours dark cycle. The animals were fed with standard pellet diet and water was given *ad libitum*. For experimental purpose the animals were kept fasting overnight but allowed free access to water.

Acute toxicity class method

Acute toxicity study was performed according to OECD guidelines 423 (Organization of Economic Co-Operation and Development). It is a step- wise procedure with three animals of single sex per step. Depending on the mortality and morbidity status of the animal, on average of 2-4 steps may be necessary to allow judgment on the test substance. The procedure is to fix a minimal number of animals, which allows acceptable database for scientific conclusion. The method uses different defined doses (5, 50, 300, 2000 mg/kg body weight) and the results allow a substance to be ranked and classified according to the "Globally Harmonized System" (GHS).

Procedure

Three healthy, Wistar Albino rats weighing 150-200 g were selected for the study. The rats were fasted over-night and provided with water *ad libitum*. Following the period of fasting, the animals were treated with the test drug at the dose of 2000 mg/kg body weight, orally. As most of the test drug possess LD_{50} value more than 2000 mg/kg body weight and this was used as starting dose. After oral administration, the rats were observed on hourly basis for 24 hours to access mortality and to detect any changes in the autonomic or behavioural responses viz. alertness, spontaneous activity, salvation, respiration, urination, aggressiveness, irritability, convulsion and corneal reflex etc. The rats were observed regularly for 14 days to note the mortality or toxic symptoms. Since there was no death as per guidelines, the study was repeated with the same dose to confirm the results. The flow charts depicts the procedure adopted for this method.

Anti Cancer Activity Study

Experimental model

For the study of anticancer activity, an experimental model is selected in such way that it would satisfy the following condition;

- The animal should develop cancer rapidly and reproducibly.
- Pathological changes in the site of induction should result from cancer formation.
- The symptoms should be ameliorated or prevented by a drug treatment effective in human beings.
- The drug tested should be administered orally.
- Drug dosage should approximate the optimum therapeutic range for human, scaled the test animal weight.

Animals

Male Swiss albino mice (20-25 gm) were produced from animal experimental laboratory, and used throughout the study. They were housed in micro nylon boxes in a control environment (temp 25±2°C) and 12 hours dark /light cycle with standard laboratory diet and water *ad libitum*. The study was conducted after obtaining institutional animal ethical committee clearance. As per the standard practice, the mice were segregated based on their gender and quarantined for 15 days before the commencement of the experiment. They were fed on healthy diet and maintained in hygienic environment in our animal house.

Technique for inducing tumour

Various techniques for induction of cancer in animals, viz., chemically induced (using DMBA/croton oil, etc.) Agarwal, 2009 virus induced, cell line induced (sarcoma – 180, ULCA fibro sarcoma and Jensen sarcoma, mouse lung fibroblast cells L-929, Dalton's Lymphoma Ascites (DAL), Ehrlich Ascites Carcinoma (EAC)(Becerra, 2006, David, 1950 and Chitra,2009) methods have been used in experimental studies of anticancer activity. In the present study, DAL cell lines induced cancer in mice was used to evaluate the anticancer activity.

Evaluation of anticancer activity

Induction of cancer using DAL cells

Dalton's Lymphoma ascites (DAL) cell was supplied by Amala cancer research center, Trissur, Kerala, India. The cells maintained in vivo in Swiss albino mice by intra peritoneal transplantation. While transforming the tumour cells to the grouped animal the DAL cells were aspirated from peritoneal cavity of the mice using saline. The cell counts were done and further dilutions were made so that total cell should be 1×10^6; this dilution was given intra peritonealy. Let the tumour grow in the mice for minimum seven days before starting treatments.

Treatment Protocol

Swiss Albino mice were divided into five group of six each. All the animals in four groups were injected with DAL cells (1×10^6 cells per mouse) intra

peritonealy, and the remaining one group is normal control group. **G1**-Served as the normal control, **G_2** - Served as the tumour control. Group 1 and 2 receives normal diet and Water, **G_3** -Served as the positive control; was treated with injection fluorouracil at 20 mg/kg body weight, Intra peritonealy, **G_4** -Served as a low dose treatment control and was administered test drug (200mg/kg, MEFNK), **G_5** -Served as a high dose treatment control and was administered test drug (400mg/kg, MEFNK), **G_6**-Served as a low dose treatment control and was administered test drug (200mg/kg, MEMNK), **G_7** Served as a high dose treatment control and was administered test drug (400mg/kg, MEMNK).

Treatment

In this study, drug treatment was given after the 24 hrs of inoculation, once daily for 14 days. On day 14, after 24 hrs the last dose, all mice from each group were sacrificed; blood was withdrawn from each mouse by retro-orbital plexus method and the following parameters were checked. **Hematological parameters**-White blood cells (WBC), Red blood cells (RBC), Hemoglobin content (Hb), Platelet count and packed cell volume (PCV). **Serum enzyme and lipid profile-** Total cholesterol (TC), Triglycerides (TG), Aspartate amino Transferase (AST), Alanine amino Transferase (ALT) and Alkaline Phosphatase (ALP). **Derived parameter-**Body weight, Life span (%) and Cancer Cell Count

Cancer cell count

The fluid (0.1ml) from the peritoneal cavity of each mouse was withdrawn by sterile syringe and diluted with 0.8 ml of ice cold Normal saline or sterile Phosphate Buffer Solution and 0.1 ml of tryphan blue (0.1 mg/ml) and total numbers of the living cells were counted using heamocytometer.

$$\text{Cell count} = \frac{\text{Number of cells} \times \text{Dilution}}{\text{Area} \times \text{Thickness of liquid film}}$$

Hematological parameters

WBC count-Total WBC count was found to be increased in cancer control, when compared with normal and treated tumor-bearing mice. The total WBC count was found to decrease significantly in animals treated with test when compared with cancer control.

RBC and Hb- RBC and Hb content decreases with tumor bearing mice when compared with Normal control mice.

Platelets-In Hodgkin lymphoma, increase in platelet count was often reported in laboratory findings. Hence, we investigated this parameter in the study.

Packed cell volume- In any case of anaemia the packed cell volume decreases.

Serum enzyme and lipid profile

Total cholesterol and triglyceride (lipid profile)- Abnormal blood lipid profile has been associated with cancer. In Hodgkin lymphoma, high cholesterol level and low triglyceride level has been reported. Hence we investigated this parameter in the study.

Liver enzymes (AST, ALT, ALP)- Abnormal liver function is seen in patients with Hodgkin lymphoma; liver enzyme levels markedly increase in tumor bearing mice. ALP is an enzyme mainly derived from the liver, bones and in lesser amount from intestines, placenta, kidneys and leukocytes. An increase in ALP levels in the serum is frequently associated with the variety of diseases. ALP comprises a group of enzyme that catalyze the phosphate esters in an alkaline environment, generating an organic radical and inorganic phosphate. Markedly elevated serum ALP, hyper alkaline - phosphatase is seen predominantly with more specific disorders; including malignant biliary cirrhosis, hepatic lymphoma and sarcoidosis. we investigated this parameter in this study.

Derived parameters

Body weight-All the mice were weighed, from the beginning to the 15th day of the study. Average increase in body weight on the 15th day was determined.

Percentage increase in life span (ILS)- % ILS was calculated by the following formula

Life span of treated group

$$\%\text{ILS} = \frac{\textit{Life span of treated group}}{\textit{Life span of control group}} - 1 \times 100$$

Effect of test drug on Survival Time

Animals were divided into five groups of six animals each. Except the normal control group, the remaining groups were inoculated with DAL cells ($1x10^6$cells/mouse) intraperitonealy on day '0' and treatment with test drug started 24 hrs after inoculation, at a dose of 5mg and 10mg/kg/day. p.o.

The normal and tumour control group was treated with same volume of 0.9% sodium chloride (NaCl) solution. All the treatments were given for fourteen days. The increase in life span (ILS) of each group, consisting of 6 mice was noted. The antitumor efficacy of test drug was compared with that of 5-fluorouracil (Dabur pharmaceutical ltd. India; 5-FU, 20 mg/kg/day, *i.p*, for 14 days). The ILS of the treated groups was compared with that of the control group using the following calculation;

Increase in lifespan = [(T - C) / C] x 100

Where,**T** = number of days the treated animal survived, **C** = number of days control animals survived.

Statistical analysis

The data's were expressed as mean ±SEM, statistical analysis was performed by one way ANOVA followed by Tukey-Kramer multiple range comparison tests, P values <0.05 were considered as significant. Highest significant test performed with software Graph pad prism 5.

RESULTS AND DISCUSSION

Preliminary Phytochemical Screening

The extracts were subjected for qualitative chemical analysis for the identification of various phytoconstituents, like alkaloids, glycosides, phenolics, flavanoids, carbohydrates, proteins and amino acids, terpenoids, sterols and saponins etc and the results of the chemical tests for each extract is recorded and tabulated in the following table-2.

Table-2 Results of qualitative phytochemical screening of petroleum ether, Benzene, chloroform, ethyl acetate, acetone and methanol of *Nymphoides krishnakesara bispinosa* male & female

S.No	Phytoconstituents	Pet. ether extract	Benzene extract	$CHCl_3$ extract	Ethyl acetate extract	Acetone extract	Methanol extract
1.	Alkaloids	-	-	++	-	-	-
2.	Glycosides	-	-	-	-	-	-
3.	Phenolics	-	-	-	-	-	-
4.	Flavones& Flavonoids	-	-	-	++	+	++
5.	Carbohydrates	-	-	-	-	-	-
6.	Proteins & Amino acids	-	-	-	-	-	-
7.	Terpenoids	-	-	-	-	-	-
8.	Sterols	-	-	-	-	-	-
9.	Saponins	-	-	-	-	-	-
10.	Gum & mucilages	-	-	-	-	-	-

Note- (++) indicate active constituents in high amount, (+) indicate active constituents in low amount (-) indicates the absence of active constituents.

IN VITRO ANTICANCER EFFECT

MTT Cell viability assay

The effects of methanol extract of plant 1(female plant), and plant 2 (male plant) on the growth of SK – MEL 28 and MCF-7 cells was examined by the MTT assay as per the method described in materials and methods. From this study, it was observed that the Methanol extract of plant 1 and Methanol extract of plant 2 at doses of 100, 200, 400 µg/ml caused marked cytotoxicity in a concentration dependent manner. The percentage viability of cells with methanol extracts of plant 1 was estimated at 92.07±0.78, 81.21±0.72, 65.35±0.38on SK – MEL 28 cells respectively and percentage viability of cells with methanol extractsof plant 2 was estimated at 84.32±1.41, 73.23±1.95, 63.65±2.18.The percentage viability of cells with methanol extractsof plant 1 was estimated at 94.30±0.71, 86.70±0.28, 68.30±0.61 on MCF – 7 cells respectively and percentage viability of cells with methanol extractsof plant 2 was estimated at 80.22±2.31, 71.42±1.80, 48.21±1.15.

The cell death was observed in methanol extract of both plants but more effect was observed in methanol extract of plant 2 than the plant 1 methanol extract. Plants at 100, 200 and 400 μg/ml concentration and the results are shown in following tables-3.

Table-3 *In Vitro* anticancer activity of methanol extracts of Plant 1 and 2

Extracts of plant1 &2	**% of cell viability SK– MEL 28**		
	Concentration μg/ml		
	100	**200**	**400**
METHANOL PLANT 1 (MEFNK)	92.07±0.78	81.21±0.72	65.35±0.38
METHANOL PLANT 2 (MEMNK)	84.32±1.41	73.23±1.95	63.65±2.18
Extracts of plant1&2	**% of cell viability MCF-7**		
	Concentration μg/ml		
	100	**200**	**400**
METHANOL PLANT 1(MEFNK)	94.30±0.71	86.70±0.28	68.30±0.61
METHANOL PLANT 2 (MEMNK)	80.22±2.31	71.42±1.80	48.21±1.15

Note-The values are expressed as mean ± S.E.M. n=3.

IN VIVO ANTICANCER EFFECT

DAL (Dalton's Ascites Lymphoma) Induced Anticancer Study of *Nymphoides krishnakesara*

ACUTE TOXICITY STUDY

During the acute toxicity study, the test drug was administered orally and animals were observed for mortality, changes in the autonomic nervous system, central nervous system and behavioural responses. There was no mortality observed even at 2000 mg/Kg for the test drug. All the animals were found to be normal and there were no behavioural changes till the end of the observation period. This observation revealed that the test drug were found to be very safe up to 2000 mg/kg of body weight known as maximum tolerated dose (MTD) by acute toxicity model study as per OECD guidelines 423. Hence from this 1/10th and 1/5th of MTD was selected and the effective doses were fixed as 200 and 400 mg/kg for further pharmacological studies.

ANTI CANCER ACTIVITY

Effect on Tumour Growth

The effect of test drug on tumour growth responses were observed and shown in Table-6. In the DAL tumour control group, the average life span of animals was found to be 49% whereas, 200 and 400 mg/kg of test drug of MEFNK showed increase in life span to 70% and 77% respectively and MEMNK showed increase life span of 72% and 81% respectively. These values were significant ($p < 0.001$) when compare with cancer control group mice. The average life span of 5- FU treated was found to be 89%, indicating its potent antitumor nature. The antitumor nature of test drug was evidenced by the significant ($p < 0.01$, $p < 0.001$) reduction of increase in body weight in animals treated with test drug at the dose of 200 and 400 mg/kg test drug MEFNK and MEMNK when compared to DAL tumour bearing mice. There was a significant ($p < 0.001$) reduction in packed cell volume and viable tumour cell count were found with 200, 400 mg/kg of test drug MEFNK and MEMNK when compared to the DAL tumour control.

Effect on Haematological Parameters

The effect of test drug on haematological parameters in cancer bearing mice was shown in Table-4. RBC count, Hb content, Platelets count were significantly ($p < 0.001$) decreased in cancer control group and were brought back to normal after treatment with 5, 10 mg/kg of test drug. WBC count was significantly ($p < 0.001$) increased in the DAL control group and was normalized by the treatment with test drug MEFNK, MEMNK at the dose of 200 and 400 mg/kg. However, the standard 5-FU at the dose of 20 mg/kg body weight produced better result in all these parameters.

Effect on Biochemical Parameters

The effect of test drug on liver, lipid profile in cancer bearing mice was shown in Table-5. In DAL inoculated mice, there was significant ($p < 0.001$) increase of the level of Total Cholesterol, Triglycerides Aspartate amino Transferase, Alanine amino Transferase and Alkaline Phosphatase when compared to the normal group. The treatment with test drug at the dose of 200 and 400 mg/kg body weight reversed these changes towards the normal level. The treatment with standard 5- FU also gave the similar results.

Table-4 Effect of test drug on Haematological parameters

Treatment	Total WBC Cells/mlx10^3	RBC Count Mill/cumm	Hb gm/dl	Platelets Lakhs/cumm
G1	9.73 ±0.85	3.15±1.15	12.45 ±2.36	3.35±0.73
G2	13.87 ±1.72^{a**}	1.65±0.95^{a**}	7.65 ±1.63^{a**}	1.86±0.57^{a**}
G3	10.13 ±1.43^{b**}	2.27±1.30^{b**}	11.6 ±1.52^{b**}	2.46±0.71^{b**}
G4	12.60±1.68^{b*}	2.18±0.68^{b*}	9.45±1.64^{b*}	1.83±0.38^{b*}
G5	11.30 ±2.05^{b**}	2.74±0.40^{b**}	10.80±1.47^{b**}	2.45 ±0.49^{b**}
G6	13.65±2.75^{b*}	2.50±0.83^{b*}	9.57±1.62^{b*}	2.15±0.35^{b*}
G7	12.72 ±2.46^{b**}	3.89±1.08^{b**}	10.69±2.07^{b**}	2.32 ±0.59^{b**}

All values are expressed as mean ± SEM for 6 animals in each group. ****a** – Values are significantly different from control (G_1) at $p < 0.001$ ***b**– Values are significantly different from cancer control (G_2) at $p < 0.01$****b**– Values are significantly different from cancer control (G_2) at $p < 0.001$

Table-5Effect of test drug on serum Enzymes and lipid proteins

Treatment	Cholesterol (mg/dl)	TG (mg /dl)	AST (U/L)	*ALT (U/L)*	ALP (U/L)
G1	100.08±2.48	125.67±4.76	38.50 ±1.73	39.25 ±1.53	130.65 ±2.78
G2	142.16±3.18^{a**}	208.65±5.10^{a**}	90.60±2.53^{a**}	61.27±1.91^{a**}	241.65±3.72^{a**}
G3	111.82±3.74^{b**}	158.23±3.61^{b**}	57.70 ±1.07^{b**}	42.69±1.26^{b**}	165.48±2.80^{b**}
G4	127.80±2.66^{b*}	188.41±1.95^{b*}	78.40 ±2.67^{b*}	56.45±2.74^{b*}	201.05±3.63^{b*}
G5	121.30±3.15^{b**}	172.35±2.72^{b**}	72.48±1.40^{b**}	44.15 ±1.46^{b**}	192.53±2.43^{b**}
G6	135.18±3.68^{b*}	184.71±4.48^{b*}	79.58 ±2.33^{b*}	50.23±1.43^{b*}	212.13±2.56^{b*}
G7	119.63±2.18^{b**}	163.82±3.43^{b**}	72.41±1.60^{b**}	43.78 ±2.58^{b**}	191.17±1.30^{b**}

All values are expressed as mean ± SEM for 6 animals in each group. ****a** –Values are significantly different from control (G_1) at $p < 0.001$, ***b**– Values are significantly different from cancer control (G_2) at $p < 0.01$, **** b**– Values are significantly different from cancer control (G_2) at $p < 0.001$

Table-6 Effect of test drug on the life span, body weight and cancer cell count of tumour induced mice.

Treatment	% ILS Life span	Increase in Body weight grams	Cancer cell count ml × 10^6 Cells/ml	PCV %
G1	>30 days	1.27±0.75	-	13.98±2.15
G2	49%	5.63±109^{a**}	2.53±0.26^{a**}	31.36±4.85^{a**}
G3	89%	2.26±0.37^{b**}	1.05±0.40^{b**}	19.75±2.74^{b**}
G4	70%	5.72±1.15^{b*}	1.76±0.54^{b*}	25.45±3.60^{b*}
G5	77%	6.33±0.97^{b**}	1.22±0.20^{b**}	21.38±2.80^{b**}
G6	72%	7.33±1.40^{b*}	1.86±0.18^{b*}	26.45±4.05^{b*}
G7	81%	6.15±0.68^{b**}	120±0.50^{b**}	22.81±2.37^{b**}

All values are expressed as mean ± SEM for 6 animals in each group. ****a** – Values are significantly different from control (G_1) at $p < 0.001$, ***b**– Values are significantly different from cancer control (G_2) at $p < 0.01$, ****b**– Values are significantly different from cancer control (G_2) at $p < 0.001$

CONCLUSION

The above study indicates positive in-vitro anticancer activity of the methanol extract *of Nymphoides krishnakesara* (whole plant) in a concentration dependent manner and In vivo pharmacological **anticancer activity** of methanolic extract of male plant show significant activity than female plant give similar result as that of 5-FU.

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