International Journal of Research in Pharmaceutical sciences and Technology

Assessment of *in-vitro* cytotoxicity and *in-vivo* antitumor activity of *Syzygium aqueum*

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ABSTRACT

The present study aims to evaluate the cytotoxicity and antitumor activity of the ethanolic extract of leaves of *Syzygium aqueum* (EESA) on K562 tumor cell line (chronic myeloid leukemia) and transplantable tumor Dalton's ascites lymphoma (DAL). In-vitro cytotoxicity on K562 tumor cell line was evaluated using micro-culture tetrazolium (MTT) assay. In-vivo antitumor activity was studied on DAL tumor bearing mice. Activity was assessed by monitoring the mean survival time, tumor cell count, percentage increase in body weight and effect on haematological parameters like red blood cell (RBC) count, haemoglobin content (Hb), white blood cell (WBC) count, Differential cell (DC) count. Histopathological studies were also evaluated during this study. EESA exhibit potent in-vitro cytotoxicity activity against K562 tumor cell line. The result also showed an increased life span and decreased tumor cell count in *S.aqueum* treated animals. The hematological studies revealed that the Hb count decreased in DAL bearing mice, whereas *S.aqueum* treated mice showed an increase in the Hb or near to normal levels and other hematological parameters such as RBC, WBC was also significantly reduced. Histopathalogical studies evaluated the destruction of tumor cells by showing cell wall destruction, membrane blebbing, degradation, vacuolated cytoplasm. The results suggested that the EESA exhibited cytotoxicity in dose dependent and it showed significant antitumor activity on DAL bearing mice.

Keywords: Antitumor; Dalton's ascites lymphoma; *Syzygium aqueum*, K562 tumor cell line.

ISSN: 2581-9143
Research Article

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Article Info
Received on: 14-09-2020
Revised on: 06-10-2020
Accepted on: 21-10-2020
DOI: https://doi.org/10.33974/ijrpst.v2i3.246

INTRODUCTION

Herbal medicine has been used as a major treatment for cancer in various countries in the Middle East and Europe a long time ago. Recent reports released by the World Health Organization (WHO) showed that although many advanced countries have considered traditional herbal treatment as an official treatment for cancer, only 5–15% of these herbs have been investigated to detect their bioactive compounds, i.e., anticancer compounds Researchers mentioned that herbal-based medicines are found to be one of the best choices for treating and/or preventing the incidence of cancer.

Cancer is a disease where an uncontrolled proliferation of cells takes place. Even though many number of anticancer drugs are available in the market, the use of chemotherapy is restricted because of their adverse reaction. Moreover, almost all anticancer drugs follow first-order kinetics, and chances of development of resistance were more. Anticancer drugs obtained from natural origin (i.e.) like Vinblastine, Vincristine, Taxol, Podophyllum were comparatively safer than other cytotoxic drugs.

Terpenoids such as xanthorhizole, Flavonoids such as myricetin, genistein, biochanin-A, Biocolein, etc. were reported for anticancer activity against many transplantable tumors.

Myricetin, a member of the flavonol class of flavonoids has an anticancer effect against several types of cancer [1] like prostate cancer, hepatocellular carcinoma, gastric cancer, and human squamous cell carcinoma [2,3,4,5]. Myricetin may also increase the chemotherapeutic potential of certain anticancer drugs by sensitizing the target cancer cells to chemotherapy [6,7].

Biochanin A, an isoflavone, is known to exert an anticancer effect on various cancer types [8,9,10]. Although not fully understood, there is some evidence that isoflavones target tyrosine

phosphorylase, topoisomerase, and drug transporters. Bio-A blocked the proliferation of glioblastoma, breast, pancreatic, and oral squamous cancer cells. Bio-A present in the diet may suppress tumor growth and be used in the prevention and combination chemotherapy that would lead to a better outcome of cancer treatment [11].

Genistein acts as a chemotherapeutic agent against different types of cancer, mainly by altering apoptosis, the cell cycle, angiogenesis, and inhibiting metastasis [12]. Genistein has been shown to suppress the growth of several cancers through modulation of various pathways [13] and they exhibited cytotoxicity against various cancer cell lines [14].

Xanthorrhizol has been known to curb the proliferation of liver cancer by inducing apoptosis through bax control and p53 in a cell [15]. Furthermore, XNT has also been known to possess antiproliferative characteristics against breast cancer MCF- 7 [16,17].

Syzygium aqueum belonging to the family Myrtaceae is a medicinal plant widely found in tropical regions such as Malaysia and Indonesia [18]. Plant *Syzygium aqueum* has been reported to possess various phytoconstituents such as flavonoids [19], tannins, and terpenoids [20]. Based on the main components of *Syzygium aqueum*, we assume/presume that *Syzygium aqueum* extract may have anticancer activities against some cancer cell lines in-vitro and in-vivo transplantable tumor model. To confirm this presume we planned to evaluate the anti-tumor activity of ethanolic extract of *Syzygium aqueum* leaves against mice model of Dalton's ascites lymphoma.

MATERIALS AND METHODS

Collection and authentication of plant part

Syzygium aqueum leaves were collected from Mahe, Pondicherry in the month of November 2017. The plant was authenticated by the Botanist, French Institute, Pondicherry and the voucher specimen was submitted in the department of Pharmacology, MTPG & RIHS for future reference (HIFP 27058).

Extraction

Syzygium aqueum leaves (50gm) were shade dried, pulverized, packed in Soxhlet extractor and extracted with ethanol (500ml) by continuous hot percolation using Soxhlet apparatus. The ethanol was removed from the extract by Distillation (solvent recovery) method. The extract was dissolved in Phosphate Buffer Saline and used for experiment.

Tumor cells

Dalton's ascites lymphoma (DAL) transplantable tumor was maintained in our laboratory through the courtesy of Amala Cancer Research Centre, Thrissur. All the animals except saline control were inoculated with 0.1ml of cell suspension of DAL (1×10^6cells/ml).

In-vitro cytotoxicity activity (MTT assay)

K562 tumor cell line (chronic myeloid leukemia) were procured from NCCS, Pune and maintained on a culture in department of Biochemistry, Pondicherry University, Puducherry. Cells (1 × 105/well) were plated in 1ml of medium/well in 24-well plates. After 48 hours incubation the cell reaches the confluence. Then, cells were incubated in the presence of various concentrations of the samples in 0.1% DMSO for 48h at 37°C. After removal of the sample solution and washing with phosphate-buffered saline (PH 7.4), 200µl/well (5mg/ml) of 0.5% 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide cells (MTT) phosphate- buffered saline solution was added. After 4h incubation, 0.04M HCl/ isopropanol were added. Viable cells were determined by the absorbance at 570nm. Measurements were performed and the concentration required for a 50% inhibition of viability (IC50) was determined graphically [21].

Percentage of cell viability was determined as $\left(\frac{\text{Avg.OD of treated cells}}{\text{Avg.OD of control cells}}\right) x\ 100$[22]

In-vivo anticancer activity

Animals

Healthy Swiss Albino Mice of 4-6 weeks old weighing between 18-25gms were procured from Biogen Laboratory animal facility, Bangalore. They were housed in polypropylene cages and maintained under standard conditions of 25±2ºC with 12h dark/light cycle. The animals were fed with standard animal pellet diet and water ad libitum. The experimental protocol was approved by the Institutional Animal Ethics Committee (CPCSEA/1923/Re/AHF/MTPG&RIHS/2ndIAEC 2018/02).

Experimental protocol

The acute and subchronic toxicity effects of the *Syzygium aqueum* leaves are evaluated and the data clearly shows there were no acute or subchronic toxicity observed [23].

All the animals except saline control were inoculated with 0.1ml of cell suspension of DAL (1×10^6cells/ml). Later these animals were grouped as follows each consisting of 10 animals:

Group I: Normal saline control; Group II: Tumor bearing mice; Group III: Ethanolic extract of *Syzygium aqueum* (200mg/kg); Group IV: Ethanolic extract of *Syzygium aqueum* (400mg/kg); Group V: -5-Fluorouracil (20mg/kg)

After 24hrs of tumor inoculation, the treatment with *Syzygium aqueum* extract was started and continued for 9 days [24]. Comparison was made between groups of mice on the fourteenth day after inoculation.

(i) **Effect of ethanolic extract of *Syzygium aqueum* on survival time**

Animals were inoculated with 1×10^6DAL cells per mouse on day 0 and treatment was started with *Syzygium aqueum*, 24 hr after inoculation, at a dose of 200 mg/kg and 400 mg/kg/ day i.p. The control group was treated with the same volume of phosphate buffer solution. All the treatments were continued for 9 days. The mean survival time (MST) of each group consisting of 10 mice was noted and the antitumor efficacy of *Syzygium aqueum* was compared with that of 5-fluorouracil (5Fu) (20mg/kg, i.p). The MST was calculated using the following formula [25]

$$MST = (1st\ death + Last\ death)/2$$

(ii) **Percentage increase in lifespan (%ILS)**

From the recorded MST, % ILS was calculated using the following formula *%ILS = (MST of respective group – MST of tumor bearing group Previous body weight) ×100*

(iii) **Effect of ethanolic extract of *Syzygium aqueum* on haematological parameters**

To determine the effect of *Syzygium aqueum* on the haematological parameters of DAL bearing mice comparison was made amongst five groups (*n=10*) of mice on the 14th day after transplantation. At the end of the experimental period, blood was collected from retro-orbital pluxes, and the red blood cell (RBC) count, haemoglobin content (Hb), and white blood cell (WBC) count was determined [26,27]. Differential cell (DC) count was carried out [25].

(iv) **Effect of ethanolic extract of *Syzygium aqueum* on tumor cell count**

The ascitic fluid was taken in a hematocrit (micro) tube and diluted 1000 times. Then a drop of the diluted cell suspension was placed on the Neubauer counting chamber and the cells were counted [28,29].

(v) **Effect of ethanolic extract of *Syzygium aqueum* on Body weight, percentage increase in body weight**

All the mice were weighed from the day of transplantation till the end of the treatment period. The Average increase in body weight was determined.

(vi) **Histopathological studies**

The DAL tumor cells from the ascitic fluid of different treatment groups were stained with hematoxylin and eosin stain and studied histopathologically

(vii) **Statistical analysis**

Statistical analysis was carried out using Graph pad prism 7.0 (Graph pad software). All results are expressed as mean± S.E.M. Groups of data were compared with the analysis of variance (ANOVA) followed by Dunnett's t-test to identify significance among groups. Values were considered statistically significant when $p < 0.05$.

RESULTS

(i) **In-vitro cytotoxicity (MTT assay)**

The effect of ethanolic extract of leaves of *S.aqueum* on K562 cell line was evaluated through micro-culture tetrazolium assay (MTT). In these studies, the cells were treated with multiple concentrations of ethanolic extract of S. aqueum (100 – 500 µg/ml). The percentage cytotoxicity increased along with concentration. The ethanolic extract of *S.aqueum* at a concentration of 500µg/ml produced 51% of cytotoxicity against K562 cell line. It was observed that the cytotoxicity is dose-dependent.

Table 1: Effect of ethanolic extract of *Syzygium aqueum* on K562 cell line

S.No	Concentration of Test Drug (µg/Ml)	Percentage Cytotoxicity (%)
1.	100	18
2.	200	24
3.	300	26
4.	400	28
5.	500	52

**Experiments were carried out only once*

In-vivo antitumor activity using DAL cell lines

(i) **Effect of ethanolic extract of *Syzygium aqueum* on survival time**

The effect of ethanolic extract of *S.aqueum* on the survival of tumor-bearing mice showed MST for the tumor control group to be 15 days, while it was 23 ± 0.83 days for *S.aqueum* extract (200mg/kg i.p), 22 ± 0.70 days for 400mg/kg and standard 5-fluorouracil (20mg/kg i.p) found to be 23.5 ± 0.32 days. The increase in the lifespan of tumor- bearing mice treated with *Syzygium aqueum* and 5-Fu was found to be 46.6%, 56.6% and 53% respectively as compared to the control group (Table 2).

(ii) **Effect of ethanolic extract of *Syzygium aqueum* on haematological parameters**

Haematological parameters of tumour bearing mice on the 15th day showed significant changes when compared with the normal mice (Table 3) and the results confirmed the rise in WBC in DAL induced animals, might be a defensive mechanism against cancer cells. Significant reduction in the raised WBC count was noticed after treatment with *S.aqueum* extract while the haemoglobin content of red blood cells count decrease

Table 2: Effect of ethanolic extract of *syzygium aqueum* leaves treatment on survival of tumor bearing mice

S.No	Treatment	Mean Survival Time (Mst)	Increase in Life Span (%)
1.	Normal control	-	-
2.	Tumor control	15 ± 1.1	-
3.	EESA (200mg/kg ip)	22 ± 0.70*	46.6
4.	EESA (400mg/kg ip)	23 ± 0.83*	>53
5.	5- Fu (20mg/kg ip)	23.5 ± 0.32*	>56.6

**Values are expressed as mean ± SEM. Number of animals used in each group = 10. Statistically, values are presented as follows: * P < 0.01 significant differences compared to tumor controls. Data were analyzed by One way ANOVA followed by Dunnett's t-test.*

Table 3: Effect of ethanolic extract of *Syzygium aqueum* on hematological parameters

S.no	Treatment	Hb (g %)	RBC (10^6/ml)	WBC (10^3/ml)	Differential count %		
					Lympho-cytes	Neutro-phils	Eosino-phils
1.	Normal control	14.2±0.45*	10.86±0.49*	8.8±0.29*	74±0.8*	25±0.84*	2 ± 0.11*
2.	DLA control	1.1±0.31*	0.65±0.03*	25.2±0.85*	85±0.63*	12.5±0.63*	2.4 ± 0.28*
3.	EESA 200mg/kg i.p	9.2±0.18*	5.60±0.31*	23.4±0.50*	84.2±0.36*	14.6±0.76*	1.4 ± 0.12*
4.	EESA 400mg/kg i.p	2.9±0.14*	1.71±0.04*	13.12±0.89*	82±0.70*	16.4±0.73*	1.6 ± 0.10*
5.	5-Fu 20mg/kg	2.0±0.14*	2.56±0.17*	9.06±0.02*	72.3±0.44*	25.4±0.96*	2.3 ± 0.17*

**p<0.01 vs. DLA control. Number of animals = 10 in each group. Statistically, values are presented as follows: * P < 0.01 significant differences compared to tumor controls. Data were analyzed by One way ANOVA followed by Dunnett's t-test.*

Table 4: Effect of ethanolic extract of *Syzygium aqueum* treatment on the tumor volume and WBC cell count

S.no	Treatment	Tumor Volume (ml)	WBC (10^6/ml)
1.	Normal control	10 ± 0.64	5 ± 0.13
2.	Tumor control	11.5 ± 0.57*	25 ± 1.15
3.	EESA 200mg/kg i.p	12 ± 0.44*	17.1 ± 0.34
4.	EESA 400mg/kg i.p	6.5 ± 0.41*	11 ± 0.90
5.	5- FU 20mg/kg	-	-

*Values are expressed as mean ± SEM. Number of animals used in each group = 10. Statistically, values are presented as follows: * P < 0.01 significant differences compared to tumor controls. Data were analyzed by One way ANOVA followed by Dunnett's t-test.*

Table 5: Effect of ethanolic extract of *Syzygium aqueum* treatment on body weight, percentage increase in body weight

Treatment	Body Weight (gm)			Percentage Increase in Body Weight (%)	
	1st Day	7th Day	14th Day	7th Day	14th Day
Normal control	19.7 ± 0.3	21 ± 0.3	22 ± 0.8	6.59	4.7
Tumor control	19.3± 1.9*	23.14± 3.4*	34.1± 6.7*	19.8	47
EESA 200mg/kg i.p	18.49± 1.8*	21.26± 1.9*	23.3± 2.2*	14.9	9.5

(iii) **Determination of tumor cell count**

The effect of *S.aqueum* was examined on the peritoneal exudate of DAL tumor mice (Table 4). Normally each mouse contain about 5×10^6/ml intraperitoneal cells [30], whereas DAL tumor bearing mice exhibited 25×10^6/ml. Upon treatment with *S.aqueum* 200 and 400mg/kg, these cell counts has been reduced to 17.1 and 11×10^6cells/ml respectively

(iv) **Effect of ethanolic extract of *Syzygium aqueum* on Body weight, Percentage increase in body weight**

Intraperitoneal inoculation of DAL cells produced a marked increase in the cancer cell count and it is represented by gain in body weight. The increase in body weight was may be due to accumulation of peritoneal fluid as suggested by an abnormal enlargement of peritoneal cavity in tumour induced mice. The decrease in the body weight & %IBW observed in the *S.aqueum* treated group indicates that it possess significant inhibitory effect on the tumour cell proliferation. The results were shown in (Table 5).

(v) **Histopathological Studies**

The DAL tumor cells from the ascetic fluid of different treatment groups were stained with hematoxylin and eosin stain. They showed marked cytological changes when compared to

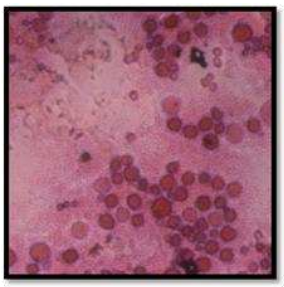

Figure 1: Smear showing DAL Tumor cells with clear cell wall without degeneration

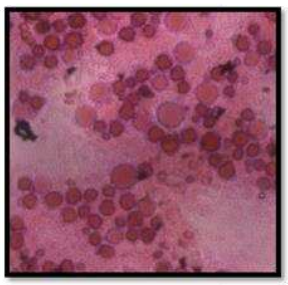

Figure 2: Smear showing DAL Tumor cells with clear cell wall and definite structure of tumor cells

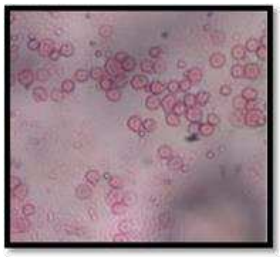

Figure 3: DAL Tumor cells of *S.aqueum* 200mg/kg treated mice, showing light reduction in staining and degradation

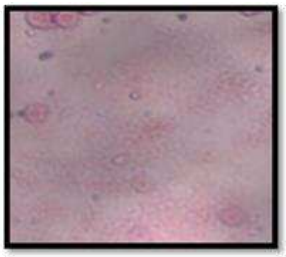

Figure 4: DAL Tumor cells of *S.aqueum* 200mg/kg treated mice, showing reduced number of cells

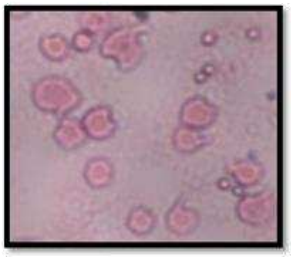

Figure 5: DAL Tumor cells of *S.aqueum* 200mg/kg treated mice, showing cell wall destruction, degradation, vacuolated cytoplasm

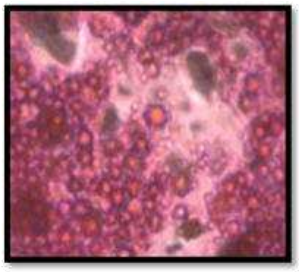

Figure 6: DAL Tumor cells of *S.aqueum* 400mg/kg treated mice, showing membrane blebbing vacuolated cytoplasm

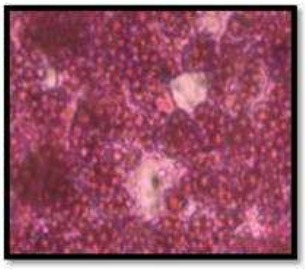

Figure 7: DAL Tumor cells of *S.aqueum* 400mg/kg treated mice, showing vacuolated cytoplasm

tumor control cells. (Figure 1; Figure 2; Figure 3; Figure 4; Figure 5; Figure 6; Figure 7).

CONCLUSION

The present study was carried out to evaluate the antitumor effect of *S.aqueum* in DAL-bearing mice. The reliable criteria for judging the value of any anticancer drugs were the prolongation of the life span of animals, reduction in tumor volume and reduction of WBC from blood [31, 32, 29]. Our results show an increase in lifespan accompanied by a reduction in WBC count in *S.aqueum* treated mice. It had significant effect in increasing the life span of ascitic tumor bearing animals. The *S.aqueum* treated animals at the doses of 200 and 400 mg/kg significantly inhibited and brought back the hematological parameters to more or less normal levels. This extract also reduced the tumor volume. To evaluate whether *S.aqueum* treatment indirectly inhibited the tumor cell growth, the effect of *S.aqueum* was examined on the peritoneal exudate of DAL tumor mice. Normally each mouse contain about 5×10^6 intraperitoneal cells [30], whereas DAL tumor bearing mice exhibited 25×10^6/ml. Upon treatment with *S.aqueum* 200 and 400mg/kg, these cell counts has been reduced to 17.1 and 11×10^6 cells/ml respectively. All these data point to the possibility of developing an ethanolic extract of *S.aqueum* as a novel, potential agent in the area of cancer chemotherapy. The phytochemical studies indicated the presence of flavonoids [19] and terpenoids [20] in *S.aqueum* extract. Flavonoids have been shown to possess antimutagenic and anti-malignant effects [33,34]. According to the previous reports, *S.aqueum* possess antioxidant activity [20]. Thus, anti-tumor effects may be due to flavonoids as well as its antioxidant activity.

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