

Research Article



Evaluation of Anticancer Properties against Ehrlich Ascites Carcinoma (EAC) Cell Line, Cytotoxic and Analgesic Activity of Methanol Extract of *Hibiscus moscheutos* in Swiss Albino Mice

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ABSTRACT

The present study has been designed to explore the analgesic, cytotoxic & in vivo anticancer activity of methanol extract of *Hibiscus moscheutos* against Ehrlich Ascites Carcinoma (EAC) in Swiss albino mice. Most of the natural sources of anticancer drugs are designed to evaluate the selective toxicity to rapidly dividing cells. The in vivo anticancer activity has been evaluated against EAC cells in Swiss albino mice by monitoring parameters including tumor cell growth inhibition, apoptosis assessment, tumor weight measurement, mean survival time and hematological parameters. This study indicated that dose of 400 mg/kg/day (i.p) that significantly increase life span, tumor cell breakdown, & decrease tumor weight, tumor cell growth rate compared to control group. Vincristine sulfate was used as a positive control (0.3 mg /kg). At the dose of 400 mg/kg/day(i.p) hematological parameters such as hemoglobin content, RBC, WBC, Lymphocytes, Neutrophils, Monocytes, Cholesterol, Triglyceride & Blood urea were significantly restored. In analgesic bioassay, the efficacy of *Hibiscus moscheutos* was 65.44% after orally administration (400 mg/kg). This outcome makes *Hibiscus moscheutos* as a possible potential candidate for using as anticancer & analgesic agent. In cytotoxic test, it showed significant result at 1479.10 µg/ml.

Keywords: Anticancer, Apoptosis, Analgesic, Cytotoxic, EAC cell line, *Hibiscus moscheutos*.

INTRODUCTION

Cancer is the second major cause of death after cardiovascular disease in the western world accounting for 24% of all deaths ¹. Free radicals are primarily responsible for the conversion of normal cell to cancerous cells as a result of a number of endogenous metabolic processes including redox enzymes, bioenergetics electron transfer and finally exposure to a plethora of exogenous chemicals. In normal metabolic condition, both the levels of oxidant and antioxidant are maintained in balance for holding optimal physiological conditions but when the production levels of free radical and reactive oxygen species (ROS) are higher than normal would attack many important biological molecules such as DNA, protein or lipid leading to many degenerative processes, such as cancer. Recently scientists have focused on medicinal plants for the investigation of anticancer agents due to the increase of cancer incidence and lack of appropriate anticancer drugs ².

In pharmaceutical industry, most of the lead compounds derived from medicinal plant along with their isolated active compounds and along with over a 100 new products are in clinical development, particularly as anti-cancer and anti-infective agents. At very early stage, chemotherapy is effective in detecting cancer but it has various side effects and resistance towards drug. For this reason, new drugs or treatments are needed. The induction of apoptosis with cytotoxic compound is known

to be an efficient and promising strategy to kill cancer cells ³. There are more than 80% of the total world's population depends on herbal medicine to fulfill their primary health care needs. Now a day, pharmaceutical industries are largely depending on natural products as a source of potential drug candidates as statistics show that over 60% of the current anticancer drugs are derived from natural medicinal plants ⁴. *Hibiscus moscheutos* is a plant that grows widely throughout the Indian subcontinent including Bangladesh and is used in folk medicine. The leaves are claimed to have multiple therapeutic properties, such as: Anti-microbial, anti-inflammatory, analgesic, sedative, cytotoxic and anticancer. For this reason this experiment was designed for the evaluation of analgesic, cytotoxic and anticancer effect of *Hibiscus moscheutos* against EAC cell line.

METHODS AND MATERIALS

Drugs and chemicals

Methanol (Merck, Germany), DMSO (Merck, Germany), Glacial acetic acid (Merck, Germany), Trypan blue dye and DAPI (Sigma-Aldrich, USA), Diclofenac sodium (Square Pharmaceuticals Ltd, Bangladesh), Sodium chloride (ACI, Bangladesh), Criston 2 (VS) (Beacon Pharmaceuticals Ltd, Bangladesh).



Plant material

Leaves of *Hibiscus moscheutos* were collected in March 2017 from Jessore -7408, Bangladesh when leaves were in their maximum densities. The plant part was thoroughly washed with water and dried in Air conditioning room (Molecular and Cell Biology Lab, JUST) at 18°C for 12 days.

Preparation of plant Extract

The dried leaves were coarsely powdered and about 1000 g of powdered material was macerated with 99.9% methanol at room temperature for a period of 10 days accompanying occasional shaking and stirring. The whole mixture was then filtered through a fresh cotton plug followed by Whatman number 1 filter paper and finally concentrated filtrate was obtained by using a rotary evaporator (Bibby RE200, Sterlin Ltd, UK) to get a viscous mass.

Experimental animal

Male *Swiss albino* mice (24-27 g) were purchased from the Department of Pharmacy, Jahangirnagar University, Savar, Dhaka 1342, Bangladesh. The animals were kept in the animal house of the Pharmacology Lab, Department of pharmacy, Jessore University of science & technology-7408, Bangladesh. The animals were kept 15 days for adaptation after collection under standard laboratory conditions (relative humidity 55%-65%, room temperature 25 °C) and maintained at natural day night cycle. The animals were provided with standard laboratory food and tap water ad libitum.

Tumor cells

EAC cells used in this study were kindly provided by Molecular Biology and Protein Science Laboratory, Department of Genetic Engineering & Biotechnology, University of Rajshahi, Rajshahi-6205, Bangladesh. Mice were injected with EAC cells by successive transplantation of 1×10^5 cells/mouse in peritoneal cavity by needle aspiration with a volume of 0.2 ml in PBS.

Determination of median lethal dose (LD₅₀)

The LD₅₀ value was determined following conventional method⁵. The test compound was dissolved in distilled water (2% DMSO) and injected intraperitoneally to six groups of mice (n=5) at different doses (100, 200, 400, 800, 1 600 and 3 200 mg/kg). LD₅₀ was calculated by recording mortality after 24 hours.

Cell growth inhibition

In vivo tumor cell growth inhibition was carried out by the method⁶. Five groups of mice (n=5) weighing 28-30 g were used for experiment. In every mouse, 1×10^5 EAC cells were inoculated into each group on day 0. Treatments were started after 24 hours of tumor inoculation and continued for six days. Group I, II and III received test compound at doses of 100 mg/kg, 200 mg/kg and 400 mg/kg (i.p.) respectively per day per

mouse. The volume of test solution was injected (i.p.) 0.2 ml/day per mouse. Group IV received VS (0.3 mg/kg, i.p.) and group V was treated with the vehicle (2% DMSO), considered as untreated control. Mice in each group were sacrificed on day seven and the total intraperitoneal tumor cells were harvested by normal saline (0.98%). Viable cells were first identified by using trypan blue and then counted by a haemocytometer. Total number of viable cells in every animal of treated groups was compared with control (EAC treated only) group. The cell growth inhibition was calculated by using the following formula:

$$\% \text{ Cell growth inhibition} = \left(1 - \frac{TW}{CW}\right) \times 100$$

Tw = Mean of number of tumor cells of the treated group of mice

Cw = Mean of number of tumor cells of the control group of mice.

Average tumor weight and mean survival time

The host survival time was recorded and expressed as mean survival time in days and percent increase of life span was calculated by method⁷. Five groups of mice (n=5) were used and 1×10^5 EAC cells were inoculated in each mouse on day 0. Treatment was started after 24 hours of tumor cell inoculation and continued for 20 days. The weight changes of each mouse were recorded on day 5, 10, 15, and 20 for such studies. Average tumor weight & mean survival time was calculated by following formulae:

The host survival time was recorded and expressed as mean survival time in days and percent increase of life span was calculated as follows¹⁶.

$$\text{Mean survival time (MST)} = \frac{\sum \text{Survival time in days of each mouse group}}{\text{Total number}} \quad \&$$

$$\text{Percent increase of life span (ILS) \%} = \left(\frac{\text{MST of treated group}}{\text{MST of control group}} - 1 \right) \times 100^*$$

Apoptosis assessment by DAPI staining

Apoptosis assessment by DAPI staining was carried out by method³. From each group of mice 1 ml of EAC cells were collected and then centrifuged at 1200 rpm for 2 min. For each time, the plate was then washed with PBS and centrifugation at 1200 rpm for 2 min for three times. The resultant cells were then incubated with 5 µl DAPI staining solution in dark for 10 min with subsequent adding of PBS to the DAPI containing pellet and then centrifuged at 1200 rpm for 2 min. Finally, 200 µL PBS was mixed with pellet and 10 µl of the supernatant was taken on a microscopic slide and observed the morphological changes of cancer cells under the fluorescence microscope (XDS-2FL, Optika, Italy).

Haematological and biochemical studies

Effect of plant extract on the haematological and biochemical parameters of EAC cell bearing mice was evaluated by the method ⁷. Five groups of mice (n=5) weighing 28-30 g were used for the experiment. In every mouse 1×10^5 EAC cells were inoculated into each group on day 0. Treatments were started after 24 hours of tumor inoculation and continued for ten days. Group I, II and III received test compound at doses of 100 mg/kg, 200 mg/kg and 400 mg/kg (i.p.) respectively (0.2 ml/day per mouse). Group IV received VS (0.3 mg/kg, i.p.) and group V treated with vehicle (2% DMSO, 5 mg/kg/mouse/day) and considered as untreated control. Then every mouse was sacrificed on 10th day and blood was collected by heart puncture. Serum was separated by centrifugation at 4000 rpm for 10 minutes and analyzed blood in a Bio analyzer (Cobas c 311 automated biochemistry analyzer, Germany).

Determination of analgesic activity

Analgesic activity of *Hibiscus moscheutos* was evaluated by using acetic acid induced writhing test in mice by method ⁸. Five groups of mice (n=5) weighing 28-30 g were used for the experiment. Control group received 1% DMSO in water at dose 10 mL/ kg, positive control received standard drug diclofenac sodium at dose 25 mg/kg and test group I, II and III received plant extract at doses 100, 200 and 400 mg/kg respectively. DMSO, standard drug and extracts were administered orally, 30 min prior to the intraperitoneal injection of 0.7% aqueous solution of acetic acid (10 ml/kg). An interval of 5 min was given for absorption of acetic acid and the number of writhing was counted for 15 min.

The percentage of inhibition:

$$\left(\frac{\text{Control mean} - \text{Treated mean}}{\text{Control mean}} \right) \times 100$$

Brine Shrimp Lethality Bioassay

Brine shrimp lethality bioassay carried out by methods ⁹.

Preparation of seawater

38 gm salt was weighed, dissolved in 1L of distilled water and filtered off to get clear solution.

Hatching of Brine Shrimp

The eggs of Brine shrimp (*Artemiasalina* Leach) were collected from an aquarium shop (Dhaka, Bangladesh). Seawater was taken in a beaker and shrimp eggs were added. Two days were allowed to hatch the shrimp and matured as nauplii. 20 nauplii were taken carefully by micropipette.

Preparation of test solutions

8.0 mg plant extract was dissolved in 200 µl of DMSO. A series of solutions of lower concentrations were prepared by serial dilution with DMSO. From each of these test solutions 100 µl were added to the pre marked glass test tubes containing 5 ml of sea water. Final concentration of

samples in the test tubes was 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250, 500, 1000 and 2000 µg/ml respectively. With the help of a Pasteur pipette 20 living nauplii were put to each of the vials ^{10, 11}.

Counting of nauplii

After 24 hours, the test tube were inspected using a magnifying glass and the number of survived nauplii in each tube was counted. The percentage mortality was calculated by dividing the number of dead nauplii by the total number, and then multiplied by 100%.

Statistical analysis

All values were expressed as mean $\pm$ SEM (n=5). Statistical analysis was performed with ANOVA followed by Bonferroni test using SPSS statistical software 16 version. The significance was set at *P < 0.05, **P < 0.01 and ***P < 0.001.

RESULTS

Median Lethal Dose (LD₅₀)

Median lethal dose (LD₅₀) of the HM was found to be 1737.80 mg/kg body weight (Figure 1).

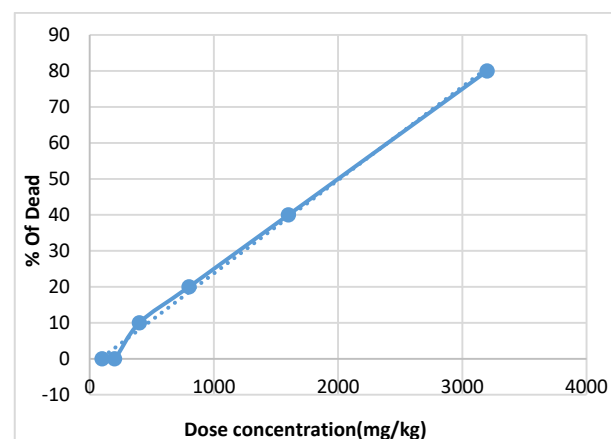


Figure 1: Median Lethal Dose (LD₅₀) calculation in Swiss albino mice.

Table 1: Effect of *Hibiscus moscheutos* on EAC cells growth inhibition considering average count of EAC cells per cell of hemocytometer (out of 16 cells).

Group	Average count of EAC cells
Control	7 $\pm$.796
Standard dose 0.3 mg/kg	1.75 $\pm$.214***
Dose 100 mg/kg	4.44 $\pm$.365**
Dose 200 mg/kg	3.56 $\pm$.456***
Dose 400 mg/kg	2.5 $\pm$.289***

Each value is represented as mean $\pm$ SEM (n = 5), significance was set at P < 0.001 (***) compare to control.



Cell growth inhibition

The average number of EAC cells growth inhibition per group that was obtained from microscope showed in the

figure 2. The test compound at dose of 400 mg/kg showed 64.3% inhibition of cell growth where standard drug VS showed 75% at dose 0.3 mg/kg (i.p) (Table 1).

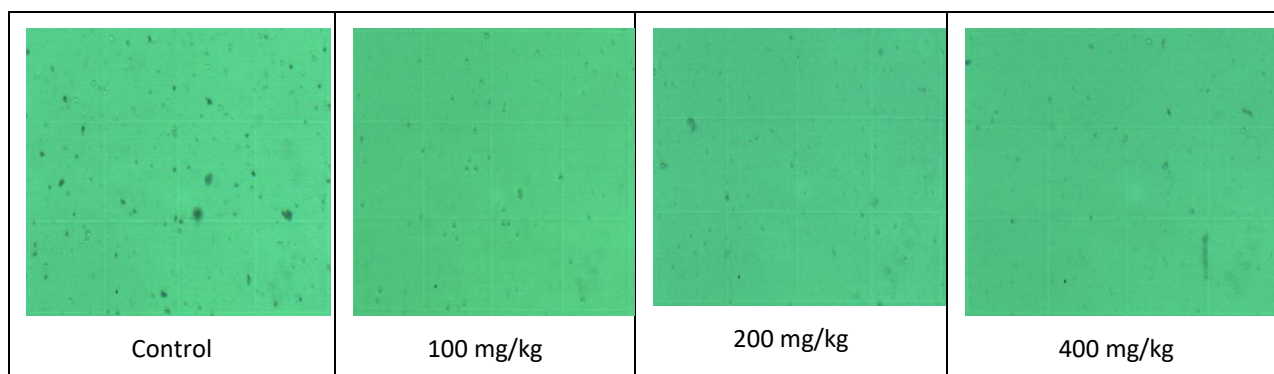


Figure 2: Growth inhibitory activity of Hibiscus moscheutos extract at different concentration on EAC cells.

Average tumor weight and mean survival time

Highest tumor weight reduction (4.13 g) was observed at the dose of 400 mg/kg (i.p) compared to the standard drug Vincristine sulfate (3.91 g) at 0.3mg/kg. At the same

time extracts at dose 100 and 200 mg/kg (i.p.) showed moderate reduction of tumor cell weight (Figure 3).

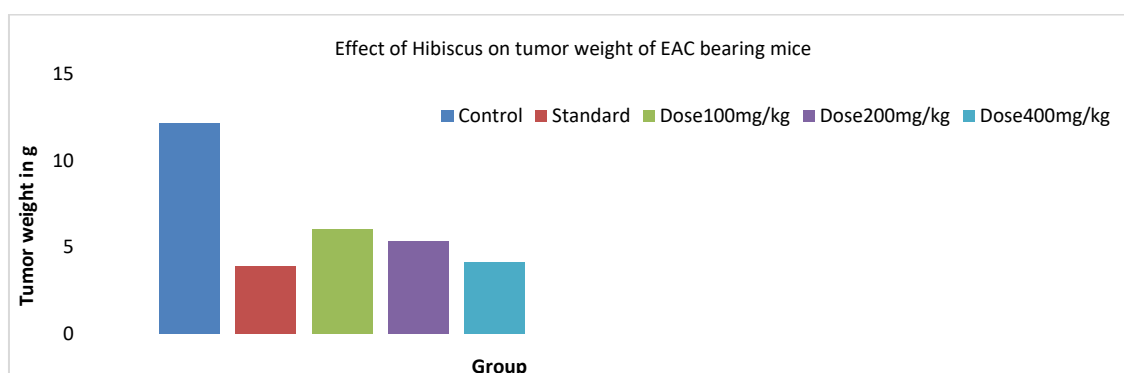


Figure 3: Effect of Hibiscus moscheutos on tumor weight of EAC cell bearing mice. Values are expressed as mean $\pm$ SEM, (n = 5); where significant value is * $p < 0.05$ & ** $p < 0.01$, Bonferroni test as compared to control.

MST of the untreated tumor bearing mice was 14 day. MST was increased remarkably after treatment with Hibiscus moscheutos. Standard drug VS showed

maximum 92.85% enhancement of life span and plant extract at dose 400 mg/kg (i.p) showed 73.80% of life span similar to standard drug (Figure 4).

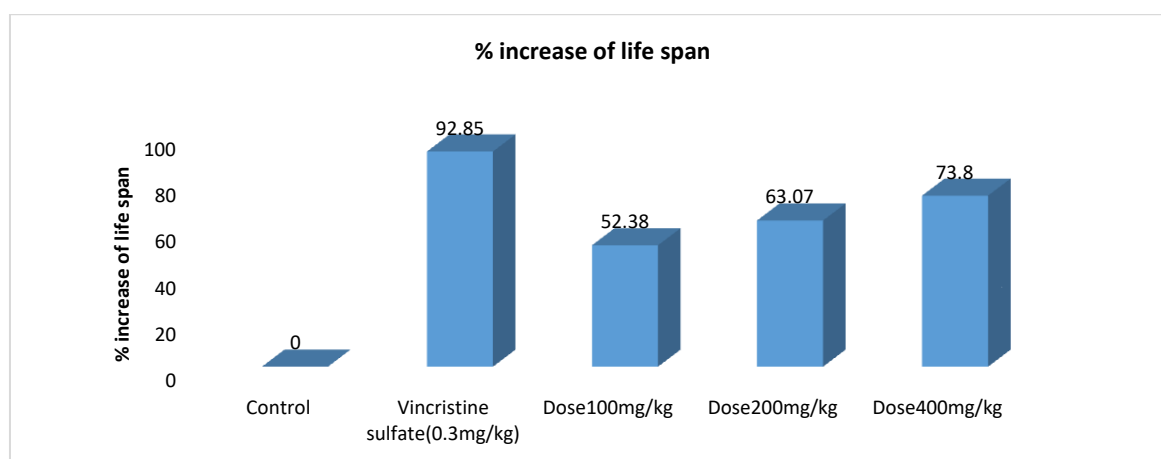


Figure 4: Effect of Hibiscus moscheutos on survival time of EAC cell bearing mice. Values are expressed as mean $\pm$ SEM, (n = 5); where significant values are, * $p < 0.01$ and ** $p < 0.001$, Bonferroni test as compared to control.

Detection of apoptotic EAC cells by DAPI staining

The apoptotic cells exhibited characteristic apoptotic changes including membrane blebbing, cell shrinkage, chromatin condensation, nuclear fragmentation and aggregation of apoptotic bodies etc. compared to normal cells. The average number of apoptotic cells per group that was obtained from fluorescence microscope showed in the figure 6. The detection of apoptotic EAC cells at dose 100, 200 and 400 mg/kg (i.p) are given in the figure 5 in comparison with control mice.

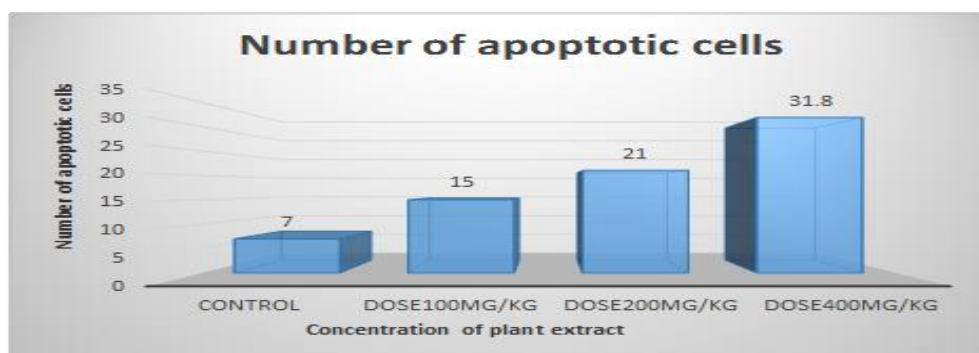


Figure 5: Detection of apoptotic cells using DAPI staining after six days of treatment. Each value expressed as mean $\pm$ SEM (n = 5). Significance was set at $p^{**}<0.01$ and $p^{***}<0.001$ Bonferroni test compared to control.

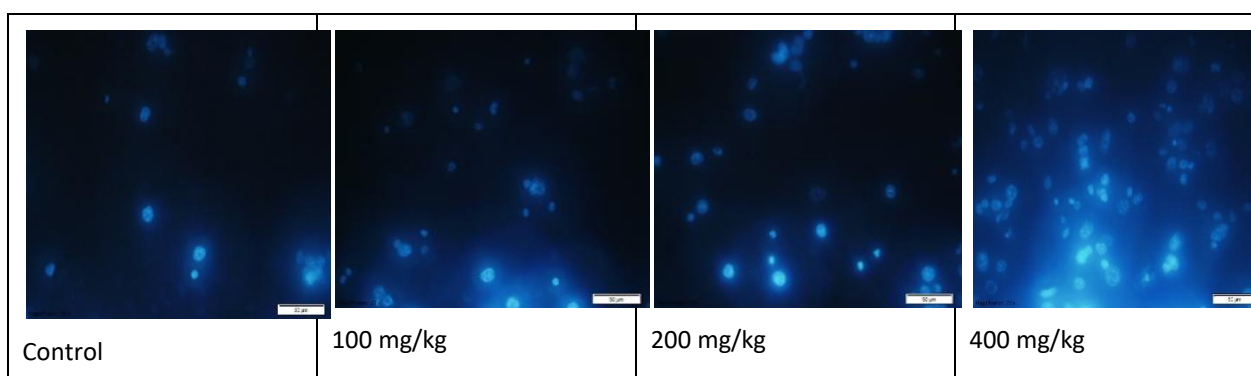


Figure 6: Detection of apoptotic cells using DAPI staining after six days of treatment at different dose of extract.

Haematological and biochemical studies

Treatment with *Hibiscus moscheutos* at doses 100, 200 and 400 mg/kg (i.p) and standard drug VS (0.3 mg/kg)

restored all haematological and biochemical parameters to normal as compared to control group. Results are shown in Table 2.

Table 2: Effect of *Hibiscus moscheutos* on haematological and biochemical parameters of EAC cell bearing mice. Each value expressed as mean $\pm$ SEM (n = 5). Significance was set at $*P<0.05$, $**P<0.01$ and $***p<0.001$ Bonferroni test compared to control.

Parameters	Normal	Control	100 mg	200 mg	400 mg	Vincristine
Hgb (g/l)	152.2 $\pm$ 1.28	74.29 $\pm$ 1.45	81.4 $\pm$.51***	98.4 $\pm$.51***	109.6 $\pm$ 0.74***	120.6 $\pm$ 1.07
RBC ($\times 10^{12}$ cells/l)	5.4 $\pm$.24	2.6 $\pm$ 0.4	2.8 $\pm$.2**	3.6 $\pm$.4**	3.8 $\pm$.37**	4.4 $\pm$ 0.24
WBC ($\times 10^9$ cells/l)	7.8 $\pm$.37	23.2 $\pm$ 1.35	20.4 $\pm$.51***	14.4 $\pm$.51***	13.8 $\pm$ 2.53***	12.4 $\pm$ 0.51
Lymphocytes (%)	71.6 $\pm$ 1.02	32.8 $\pm$ 1.02	39.6 $\pm$.67***	46.8 $\pm$.58***	54.6 $\pm$ 1.20***	61.8 $\pm$ 0.66
Neutrophils (%)	18.2 $\pm$.58	61.8 $\pm$ 1.35	53.8 $\pm$.73***	49 $\pm$.94***	43.8 $\pm$ 0.73***	32.8 $\pm$ 0.8
Monocytes (%)	2.6 $\pm$.40	1.4 $\pm$ 0.24	2.2 $\pm$.2*	1.8 $\pm$.2*	1.6 $\pm$ 0.4*	2.8 $\pm$ 0.49
Cholesterol (mg/dl)	105.4 $\pm$ 1.20	151.2 $\pm$ 0.58	143.4 $\pm$.67***	139.4 $\pm$.4***	131.8 $\pm$ 0.8***	121.8 $\pm$ 1.15
Triglyceride (mg/dl)	101.4 $\pm$ 1.53	183.8 $\pm$ 0.73	172.4 $\pm$.67***	164 $\pm$.74***	155.6 $\pm$ 0.872***	133.8 $\pm$ 0.73
Blood urea (mg/dl)	22.8 $\pm$.97	62.6 $\pm$ 0.67	53.8 $\pm$.66***	46.4 $\pm$.6***	39.8 $\pm$ 0.73***	32.8 $\pm$ 0.73

Analgesic activity

The test compound at dose 400 mg/kg produced 65.44% writhing inhibition where standard drug Diclofenac sodium produced 77.07% writhing inhibition in mice. The results are shown in Table 3.

Groups	Number of writhes	Inhibition rate (%)
Control	60.2±.58	00.00
Diclofenac sodium	13.8±.66	77.07***
Dose 100mg/kg	39.6±.81	34.21**
Dose 200mg/kg	29.6±.74	50.83***
Dose 400mg/kg	20.8±.58	65.44***

Table: 3. Effects of *Hibiscus moscheutos* on acetic acid-induced writhing in mice. Values are expressed as Mean±SEM where n=5. Significance was set at ***p<0.001 Bonferroni test compared to control.

Brine shrimp lethality bioassay (LC₅₀)

Median lethal concentration (LC₅₀) of brine shrimp lethality was found to be 1479.10 µg/ml that are shown Figure 7.

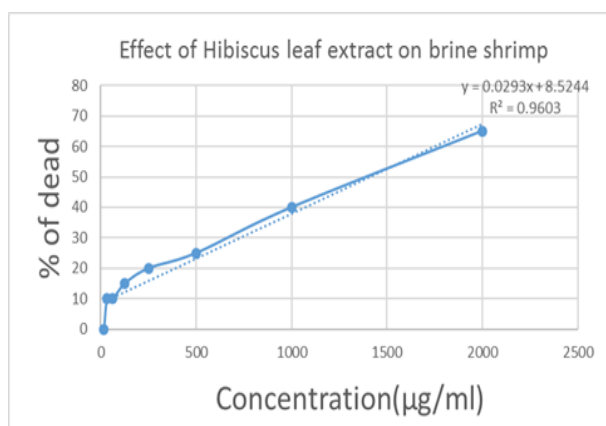


Figure 7: Effect of plant extract on brine shrimp nauplii after 24 hrs. Of incubation (n=20).

DISCUSSIONS

As anticancer agent, the effectiveness of HM has been showed by measuring the inhibition of cell growth, breakdown of cancer cells, reduction in tumor weight and enhancement of MST & restoring of hematological and biochemical parameters of the EAC cell bearing mice ¹². The efficiency of the test compound has been compared with a known effective anticancer drug, VS at the dose of 0.3 mg/kg (i.p.).

For EAC bearing mice, the tumor weight has been found to increase rapidly. The treatment of such mice with test compound reduced the growth rate. At the dose of 400mg/kg of HM showed significant cell growth inhibition by 64.3% where known drug VS showed 75% at the dose of 0.3mg/kg (i.p.), (Figure 2) ⁷.

Apoptosis is a process of cell death in where the body selectively eliminates unnecessary cells or unhealthy cells

without affecting surrounding normal cell. When apoptosis will be inhibited tumor will be developed which allows the cell to proliferate abnormally and leading to the development of cancer. Therefore, induction of apoptosis is considered as a central strategy and a useful indicator for almost every type's cancer treatment and prevention. At the dose of 400 mg/kg (i.p) of HM showed the mean number of apoptotic cells 31.8 where EAC cell bearing mice showed 7 ³.

The life span of the EAC cell bearing mice increased & reduced tumor weight remarkably when treated with the test compound. At the dose of 400 mg/kg (i.p) of HM has increased life span 73.80% when VS at the dose 0.3mg/kg showed 92.85%. At the same dose of HM (i.p) reduced tumor weight 4.13 g when VS at 0.3mg/kg showed 3.91g. The prolongation of the life span & reduction of tumor weight of cancer bearing mice is a very important and reliable criterion for judging the potency of any drug as anticancer agent. The effectiveness of the compound against EAC cell bearing mice has further been verified by monitoring the change in hematological and biological parameters ⁷.

This study indicates that the number of cell growth decreased and number of apoptotic cells increased significantly at different doses. All these are measured are very important aspects in justifying the effectiveness of a compound in cancer chemotherapy ¹³.

In cancer chemotherapy the major problem is anemia. The anemia occur in tumor bearing mice is mainly due to a reduction in RBC or hemoglobin percentage and this may occur either due to iron deficiency or due to hemolytic or myelopathic conditions.

At the dose of 400 mg/kg (i.p) of HM significantly recover the hemoglobin content, RBC and WBC cell count that indicates the protective action of HM on the haemopoietic system. Hypoglycemia and hyperlipidemia occur in experimental animals with carcinoma. In this experiment, the reduced glucose level and elevated cholesterol, triglycerides and serum urea were restored to more or less normal levels in HM-treated mice, thereby indicating a potent anticancer efficacy of HM ⁵. An analgesic is an agent that selectively relieves pain by acting in the central nervous system or on peripheral pain mechanisms ¹⁴, without significantly altering consciousness ⁸. At the dose of 400 mg/kg of HM produced 65.44% writhing inhibition where standard drug Diclofenac sodium produced 77.07%.

Median lethal concentration (LC₅₀) of brine shrimp lethality was found to be 1479.10 µg/ml that is active & nontoxic. LC₅₀ value of less than 1000 µg/mL is toxic while LC₅₀ value of greater than 1000 µg/mL is non-toxic ^{11,15}.

CONCLUSION

The present inquiry is amply incentive as it pursue that *Hibiscus moscheutos* have potential analgesic and



anticancer activity. Finally our verdict point out that the methanol extract of *Hibiscus moscheutos* leaf indicates potential anticancer and analgesic activities which further turn out as a novel source of phytomedicines in the field of analgesic, free radical and also in cancer biology. Moreover, further investigation require to identify the active principle involved for demonstrating anticancer, analgesic, and antioxidant activity.

Abbreviation

HM= *Hibiscus moscheutos*, EAC=Ehrlich Ascites Carcinoma, NCI=National Cancer Institute, i.p.=intraperitoneally, µg/ml=Microgram per milliliter, mg/kg=milligram per kilogram, Hgb=Hemoglobin, WBC=White blood cell, RBC=Red blood cell, SEM=Standard error mean, %=percentage, ANOVA=one way analysis of variance, DAPI=4', 6-diamidino-2-phenylindole, DMSO=Dimethyl sulfoxide, PBS =phosphate buffered saline, rpm=rotation per minutes, VS=Vincristine sulfate, MST= Mean survival time, JUST= Jessore University of Science & Technology.

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Conflict of Interests: The authors declare that there is no conflict of interests regarding the publication of this paper.

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