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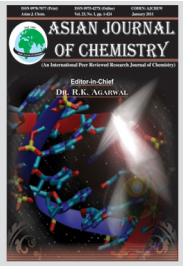

# Antioxidant Activity of the Fractions of *Cleome gynandra* Promotes Antitumor Activity in Ehrlich Ascites Carcinoma

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The plant *Cleome gynandra* traditionally used as an Indian medicine to treat microbial diseases. It's anticancer and antioxidant activity is already proved by our research groups; therefore we started to perform column chromatography of methanol extract over silica-gel. Four flavonoid fractions were isolated with mixture of minor compounds and administered at the doses of 150 mg/kg body weight intraperitoneally for 9 consecutive days. Twenty four hours of last dose and 18 h of fasting, the mouse were sacrificed and antitumor effect of all the fractions were assessed by evaluating tumor volume, viable and nonviable tumor cell count, tumor weight, biochemical, enzymatic and hematological antioxidant parameters of EAC bearing host. The high amount of antioxidant flavonoid is responsible for significant antitumor activity which correlates its *in vitro* cytotoxic effect. But the antitumor and cytotoxic activity shows synergistic action along with saponin.

**Key Words: *Cleome gynandra*, Coloum chromatography, EAC cell line, Antitumor activity, Antioxidant activity, 5-Fluorouracil.**

## INTRODUCTION

Cancer (medical term: malignant neoplasm) is a class of diseases in which a group of cells display uncontrolled growth (division beyond the normal limits), invasion (intrusion on and destruction of adjacent tissues) and sometimes metastasis (spread to other locations in the body *via* lymph or blood). These three malignant properties of cancers differentiate them from benign tumors, which are self-limited and do not invade or metastasize. Cancer may affect people at all ages, even fetuses, but the risk for most varieties increases with age[1].

Flavonoids have been shown to posses antimutagenic and antimalignant effect[2]. Further more, flavonoids have a chemopreventive role in cancer through their effect on signal transduction in cell proliferation and angiogenesis[3]. The high content of phenolic compounds are known to have direct antioxidant property due to presence of hydroxyl groups which can function as hydrogen donor[4]. The oxidative stress has also been implicated in the pathogenesis of cancer. Many plants contain substantial amounts of antioxidant mainly flavoniod compound and can be utilized to scavenge the excess free radicals from human body. It is reasonable to expect that high antioxidant potential to reduce free radicals in the body is due to the total phenol compound in the plant.

The plant *Cleome gynandra* L, (Capparidaceae) is commonly known as 'Hurhur'and 'Karaila' in India and 'Cat's whiskers' in English[5]. The leaves and seeds of cat's whiskers are used in indigenous medicine in many countries. Cat's whiskers grow as a weed in most tropical countries. It has been used for several years in Indian traditional medicine as an anthelmentic and antimicrobial agent[6]. Leaves are applied externally over the wounds to prevent the sepsis. The decoction of the root is used to treat fevers. The methanolic extract of *Cleome gynandra* possess good free radical scavenging and also antioxidant property[7,8]. The leaves are used as disinfectants. Inhalation of the leaves also relieves headaches; leaf juice and oil, for earache and eye wash. Seeds have been reported to have antihelmintic properties and oil is used as fish poison. Stems are used as analgesic and antiinflammatory agent[9]. The whole plant is also used in the treatment of malaria, piles, rheumatism and in tumor[10].

The anticancer activity of the methanol extract of *Cleome gynandra* against Ehrlich's Ascites Carcinoma cells bearing mice was already proved by our research groups[11]. The present study evaluated that the antitumor effect of the fractions of methanol extract of *Cleome gynandra* against Ehrlich's Ascites Carcinoma (EAC) cell in Swiss *albino mice*. Fractionation was done by continuous coloum chromatography over

silica gel and eluted with increasing polarity. All the fractions were monitored by thin layer chromatography using precoated aluminum plates (Emark). Four major flavonoid fractions were isolated along with mixture of minor compounds.

## EXPERIMENTAL

**Preparation of the extract:** Plant was collected from Jalpaiguri, West Bengal, India in January 2009 and identified by the Botanical Survey of India, Botanic Garden, Howrah, India. A voucher specimen (No.-CNH/I-I/(293)/2009/Tech.II/335) has been preserved at our laboratory for future reference.

Air dried whole plant (500 g) material except roots were powdered in a mechanical grinder and the plant material were successively extracted by chloroform and methanol by using soxhlet extraction apparatus. Then solvent was completely removed under reduced pressure and stored in a vacuum dessicator. The yield of the chloroform and methanol fractions were about 7.25 and 9.30 %, respectively. The methanolic extract used for the fractionation by column chromatography.

Sodium chloride, propylene glycol, iodine, trichloro acetic acid, thiobarbituric acid, EDTA, DTNB, Trypan blue, methyl violet, sodium sulphate, methylene blue, 5-fluorouracil and all other chemicals, solvents, enzyme kits and reagents used were of highest analytical grade. TLC plate from (MERK-TLC silica gel 60 $F_{254}$, 20 cm × 20 cm) MERK Mumbai India.

**Phytochemical screening:** Phytochemical study of the methanol extract was done by method of Pollock and Steven[12] and study indicated the presence of flavonoid, alkaloids, tannins and saponin. Total phenolic compound was measured by Folin-Ciocalteu's phenol reagents[13]. Furthermore flavonoid was also confirmed by magnesium-metal test[14].

**Isolation and purification of the fractions:** Column chromatography was performed over Si-gel 60-120 mesh[15]. Elution was performed with solvent of increasing polarity, starting with chloroform (called isocratic elution). Gradient elution is performed by adding methanol in chloroform in different extent. Total 130 (50 mL in each fraction) fraction was collected and subsequent TLC is performed to evaluate the solvent system in the column. All fractions collected from the column using 50 % methanol in chloroform were mixed and marked as FlavSt-1. The phytochemical screening of this fraction shows the presence of flavonoid and steroid.

The remaining fractions were marked and TLC was performed by using 18:2:1:4 (chloroform:methanol:ehtylacetate: benzene) solvent system and all flavonoid fractions were mixed and marked as FlavSt-2. Phytochemical analysis indicate the presence of flavonoid along with steroids.

Purification of the remaining half mixture was performed by column chromatography over Si-gel 100-200 mesh. Elution was performed with solvent of increasing polarity, starting with petroleum ether. Isocratic elution was performed using 100 % petroleum ether then gradient elution started with 10 % chloroform and upto 75 % chloroform in petroleum ether. Total 40 fraction (25 mL in each fraction) were collected further with increasing polarity by increasing the chloroform part in the the mixture as because of the higher polarity of the chloroform than the petroleum ether in the mixture. Similar fraction was mixed according to the $R_f$ value and chemical test. Fraction number 50-60 was chromatographed by using precoated TLC plate ( MERK-TLC silica gel 60 $F_{254}$, 20 cm × 20 cm) with the solvent system 18:3:0:4 (chloroform:methanol: ehtylacetate: benzene) which give 4 spots but single spot with 6:3:1 (benzene:chloroform:ehtylacetate) with a $R_f$ value of 0.75. The phytochemical screening of this fraction were done which shows presence of flavonoid and saponin. All the fractions were mixed and marked as FlavSap-3.

Then again isocratic elution was performed by using 100 % chloroform and subsequent gradient elution by adding methanol in chloroform (increasing polarity). 70-Fractions (25 mL in each) were collected and subsequent chromatography was performed. Then all fractions of 50 % methanol in chloroform were mixed and marked as FlavSap-4 for further anticancer activity as because it contain flavonoids with minor mixture of saponin.

**Identification and chromatographic characterization of the fractions:** All of the eluted fractions were monitored by phytochemical screening and by TLC using precoated aluminum plates. Presences of flavonoid were confirmed by magnesium metal test[12]. All other phytochemical test were performed by the methods of[13,14]. Four major flavonoid fractions along with some minor compounds were selected and characterization was performed by thin layer chromatography[15].

**Toxicity study:** Hemolytic activity of the four fractions towards murine erythrocytes was tested. At least 1 % washed erythrocyte suspension (in PBS 0.02 M, pH 7.4) was incubated with the different concentration of all the fractions at 37 °C for 45 min. Distilled water and normal saline were taken as positive and negative controls, respectively. After incubation, the erythrocytes were separated and the amount of hemoglobin released was determined by measuring the absorbance of the supernatant at 540 nm. Percentage death were calculated by assuming that all the cells were dead in positive control and all cells were alive in negative controls[16].

**Animals:** Male Swiss *albino mice* weighing 20-22 g were taken. They were obtained from B.N. Ghosh & Co. Kolkata, India. The mice were grouped and housed in poly acrylic cages (38 cm × 23 cm × 10 cm) with not more than 6 animals per cage and maintained under standard laboratory conditions (temperature 25 ± 2 ºC and dark/light cycle 14/10 h). They were allowed free access to standard dry pellet diet (Hindustan Lever, Kolkata, India) and water *ad libitum*. The mice were acclimatized to laboratory conditions for 7 days before commencement of the experiment. All procedures described were reviewed and approved by the University Animal Ethics Committee.

**Transplantation of tumor:** EAC cells were obtained from Chittaranjan National Cancer Institute (CNCI), Kolkata, India. The EAC cells were maintained *in vivo* in Swiss albino mice by intraperitoneal transplantation of $2 \times 10^6$ cells per mouse after every 10 days. Ascitic fluid was drawn out from EAC tumor bearing mouse at the log phase (day 7-8 of tumor bearing) of the tumor cells. Each animal received 0.1 mL of tumor cell suspension containing $2 \times 10^6$ tumor cells intraperitoneally.

**Treatment schedule:** 140-Swiss albino mice were divided into seven groups (n = 20) and given food and water *ad libitum*.

All the animals in each groups received EAC cells ($2 \times 10^6$ cells/mouse i.p.) except group-I. This was taken as day '0'. Group-I served as normal saline control (5 mL/kg i.p.) and group-II served as EAC control. 24 h after EAC transplantation, group-III to VI received different fraction at the dose of 150 mg/kg i.p. for 9 consecutive days. Group-VII received reference drug 5-FU (20 mg/kg i.p) for 9 consecutive days[16,17]. Twenty four hours of last dose and 18 h of fasting, 10 animals of each group were sacrificed by cervical dislocation to measure antitumor activity, biochemical, hematological parameters, enzymatic assay and rest were kept with food and water *ad libitum* to check percentage increase in life span of the tumor host. The antitumor activities of the fractions were measured in EAC animals with respect to the following parameters:

**Tumour volume:** The ascitic fluid was collected from the peritoneal cavity. The volume was measured by taking it in a graduated centrifuge tube.

**Tumour weight:** The tumor weight was measured by taking the weight of the mice before and after the collection of the ascetic fluid from peritoneal cavity.

**Percentage increase in life span (ILS):** The percentage increases in life span was calculated on the basis of mortality of the experimental mice[18,19].

ILS (%) = [(Mean survival time of the treated group/mean survival time of the control group)-1] × 100

$$\text{Mean survival time} * = \frac{[\text{First Death} + \text{Last Death}]}{2}$$

*Time denoted by days.

**Tumor cell count:** The ascitic fluid was taken in a WBC pipette and diluted 100 times. Then a drop of the diluted cell suspension was placed on the Neubauer's counting chamber and the numbers of cells in the 64 small squares were counted.

**Viable/nonviable tumor cell count:** The viability and nonviability of the cell was checked by trypan blue assay. The cells were stained with trypan blue (0.4 %in normal saline) dye. The cells that didn't take up the dye were viable and those that took the dye were nonviable. These viable and nonviable cells were counted.

Cell count = Number of cells x dilution factor/Area × thickness of liquid film.

**Hematological assay:** At the end of the experimental period, the next day after an over night fasting blood was collected from freely flowing tail vein and used for the estimation of hemoglobin (Hb) content, red blood cell (RBC) count, white blood cell (WBC) count by standard procedures.

**Enzymatic assay:** 1-G of liver tissue was collected wash with nomal saline, soaked in filter paper. Homogenised in 10 mL of 0.15 M tris buffer pH-7.4 and centrifused at 3000 g at 4 ºC for 0.5 h. Supernatant was collected and different antioxidant and pro-oxidant marker were performed using biochemical test[1,17]. Total malondialdehyde were measured as a product of lipid peroxidation[20]. Reduce glutathione level and catalase activities were also measured by some biochemical methods[21,22].

**Biochemical assay:** 24 h of last dose and 18 h of fasting, ten animals of each group were sacrificed by cervical dislocation to collect the blood from freely following tail vein of the mouse. The blood sample were then allowed to clot and the serum was separated by centrifugation at 2500 g at 37 °C and are used for to performed the serum marker using biochemical methods[1,17]. Serum glutamate-oxaloacetate transaminase (SGOT), serum glutamate-pyruvate transaminase (SGPT) and Serum alkaline phosphatase (SALP) are the principle serum marker which were measured by biochemical reaction[23,24].

***In vitro* cytotoxicity study of the fractions:** *In vitro* cytotoxicity of all the four fractions was done against Ehrlich Ascites Carcinoma cell. The EAC cells were collected counted and adjusted $10^6$ cells/mL with phosphate buffer solution (0.2 M, pH-7.4). Various concentrations of the each fraction were added and incubating in 37 °C for 3 h. The viability of the cells were determined by trypan blue exclusion methed[25].

**Statistical analysis:** All data are expressed as mean ± SEM (n = 10 mice per groups). Statistical significance (p) calculated by one-way ANOVA between the treated groups and the EAC control followed by Dunnett's post hoc test of significance where $p < 0.05$ and $p < 0.01$ considered to be significant and highly significant, respectively.

## RESULTS AND DISCUSSION

In the hemolytic activity study only 1.4 ± 0.05 and 1.7 ± 0.08 % of murine erythrocytes were dead in case of FlavSap-3 and FlavSap-4, respectively at a concentration of 0.5 mg/mL. Where in case of FlavSt-1 and FlavSt-2 it lyses around 2.89 ± 1.02 and 3.05 ± 1.25 %, respectively at same concentration. 50 % inhibitory concentrations were found to be 6.5 ± 1.2, 6.8 ± 1.9, 7.5 ± 2.2 and 8.5 ± 2.1 % mg/mL in case of FlavSt-1, FlavSt-2, FlavSap-3 and FlavSap-4, respectively. So all the fractions is almost safe in mice erythrocyte.

The *in vitro* cytotoxicity study showed that the fraction FlavSap-3 and FlavSap-4 are more cytotoxic than the fractions FlavSt-1 and FlavSt-2. $IC_{50}$ value of the all the fractions were 800 ± 5.68, 750 ± 6.78, 25 ± 5.51and 20 ± 4.55 µg/mL for FlavSt-1, FlavSt-2, FlavSap-3 and FlavSap-4, respectively. But $IC_{50}$ value of methanol and chloroform extract is 50 ± 8.21 and 850 ± 6.32 µg/mL, respectively. It shows that FlavSt-1 and FlavSt-2 is highly cytotoxic than the chloroform extract but FlavSap-3 and FlavSap-4 are more cytotoxic than both the methanol and chloroform extract.

Intraperitoneal administration of all the fractions at a dose of 150 mg/kg body weight increased the life span (ILS) and nonviable cell count, when compared to that of EAC control mice. The mouse transplanted with the EAC cells exhibited a constant weight gain and increase in the volume due to tumor cell multiplication and growth. Administration of the fraction FlavSap-3 and FlavSap-4 significantly ($p < 0.01$) reduce the tumor weight, tumor volume and viable cells count and that is comparable with standard 5-FU (Table-1).

Tumor induction increased total number of WBC by nearly four folds. Administration of the fractions FlavSt-1 and FlavSt-2 significantly ($p < 0.05$) reversed the tumor induced rise in total counts of WBC. But the WBC levels restored towards normal level more significantly ($p < 0.01$) by the treatment of the fractions FlavSap-3, FlavSap-4 and 5-FU (Fig. 2C). The number of RBC count and hemoglobin content also increased (Fig. 2A and 2B) in such a way that the fractions FlavSap-3,

TABLE-1
EFFECT OF THE FOUR FRACTIONS ON TUMOR VOLUME, TUMOR WEIGHT, MEAN SURVIVAL TIME (MST), PERCENTAGE INCREASE LIFE SPAN (ILS %), VIABLE AND NONVIABLE TUMOR CELL COUNT IN EAC BEARING MICE. EACH POINT REPRESENTS THE MEAN ± SEM (n = 10 MICE PER GROUPS). *$p < 0.05$, **$p < 0.01$ AND 'a' INDICATE THAT NOT SIGNIFICANT WHEN TREATED IS COMPARED WITH CONTROL

| Groups | Viable cell count | Nonviable cell count | Tumor volume (mL) | Tumor weight (g) | Packed cell volume (mL) | ILS (%) |
|---|---|---|---|---|---|---|
| Control | $8.54 \times 10^7 \pm 0.46$ | $0.6 \times 10^7 \pm 0.04$ | $2.8 \pm 0.15$ | $3.61 \pm 0.33$ | $1.96 \pm 0.14$ | 0.00 |
| FlavSt-1 | $6.92 \times 10^7 \pm 0.93^{*}$ | $0.9 \times 10^7 \pm 0.30^{a}$ | $1.7 \pm 0.21^{*}$ | $1.52 \pm 0.28^{*}$ | $1.30 \pm 0.20^{*}$ | 14.6 |
| FlvSt-2 | $4.33 \times 10^7 \pm 0.55^{**}$ | $1.2 \times 10^7 \pm 0.36^{*}$ | $1.4 \pm 0.23^{**}$ | $1.53 \pm 0.27^{*}$ | $0.80 \pm 0.15^{**}$ | 39.0 |
| FlavSap-3 | $2.22 \times 10^7 \pm 0.70^{**}$ | $2.9 \times 10^7 \pm 0.72^{**}$ | $1.2 \pm 0.39^{**}$ | $1.33 \pm 0.18^{*}$ | $0.66 \pm 0.16^{**}$ | 76.0 |
| FlavSap-4 | $2.06 \times 10^7 \pm 0.38^{**}$ | $3.2 \times 10^7 \pm 0.63^{**}$ | $1.1 \pm 0.12^{**}$ | $0.90 \pm 0.13^{**}$ | $0.56 \pm 0.03^{**}$ | 82.5 |
| 5-FU | $0.81 \times 10^7 \pm 0.05^{**}$ | $3.5 \times 10^7 \pm 0.05^{**}$ | $0.5 \pm 0.21^{**}$ | $0.48 \pm 0.12^{**}$ | $0.45 \pm 0.05^{**}$ | 102.4 |

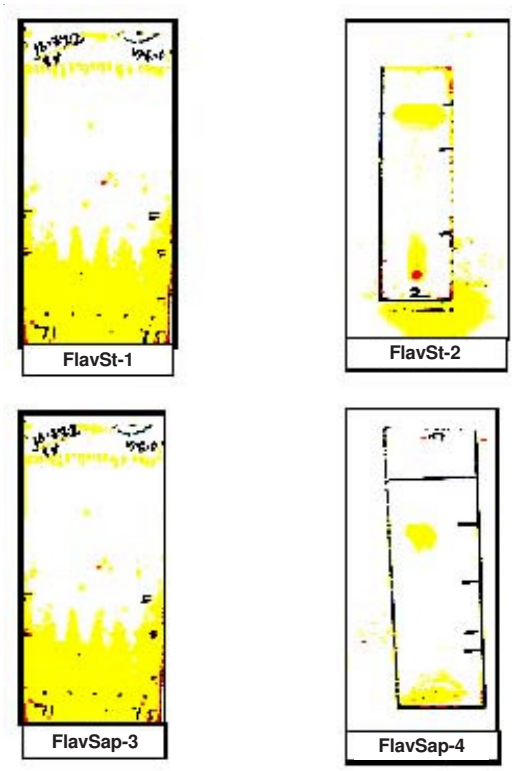



Fig. 1. Thin layer chromatogram of the fractions using MERK-TLC silica gel 60 $F_{254}$, 20 cm × 20 cm plate and iodine chamber as a detecting reagent. FlavSt-1(flavonoid with mixture of steroid): $R_f$ (retardation factor) = 0.66, 0.53, 0.36, 0.92 in 50:50 (chloroform:methanol). FlavSt-2 (flavonoid with mixture of Steroid): $R_f$ = 0.81, 0.50, 0.27, 0.18 in 18:3:1:3 (chloroform:methanol:ehtylacetate:benzene). FlavSap-3 (flavonoid with mixture of saponin): $R_f$ = 082, 0.24, 0.36, 0.23 (18:3:1:3 = chloroform:methanol:ehtylacetate:benzene). FlavSap-4 (flavonoid with mixture of saponin): $R_f$ = o.63, 0.38 in 50:50 (chloroform:methanol)

FlavSap-4 and 5-FU show more significantly value than the fractions FlavSt-1 and FlavSt-2. So all the fraction more or less restored the hematological parameters.

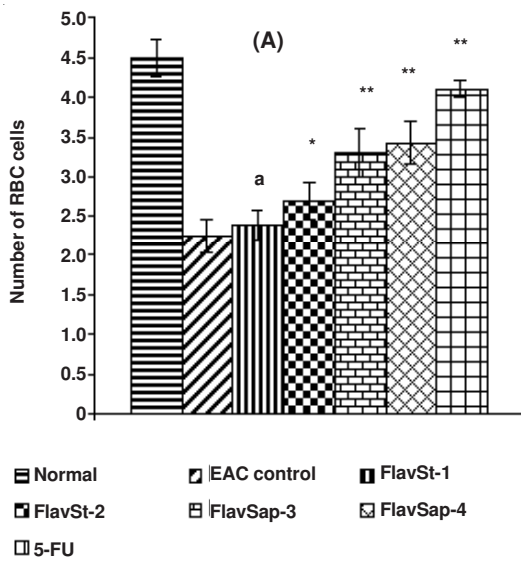
Antioxidant enzymes namely catalase (CAT) and glutathione reductase profiles were estimated spectrophotometrically as per the mention protocol. The extent of lipid peroxidation in terms of malondialdehyde (MDA) formation was measured accordingly. In the present study, all the fractions restored the antioxidant enzyme level towards more or less normal (Fig. 3).

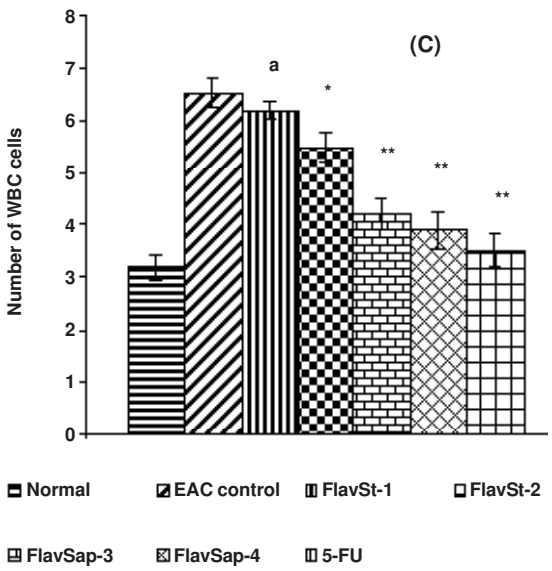
Biochemical parameters regarding serum glutamate-oxaloacetate transaminase (SGOT), serum glutamate-pyruvate transaminase (SGPT) and serum alkaline phosphatase (SALP) also reduced towards normal level by the treatment of the all fractions. 5-FU as well as the FlavSap-3 and FavSap-4 reversed these changes significantly ($p < 0.01$) than other two fractions (Fig. 4).

Most of the biologically active constituents of plants are soluble in polar solvent or water. Water soluble phytoconstituents (like flavonoids) are poorly absorbed either due to their large moleculer size which can not absorb by passive diffusion, or due to their poor lipid solubility; severely limiting their ability to pass across the lipid-rich biological membranes, resulting poor bioavailability. It has often been observed that the isolation and purification of the constituents of an extract may lead to a partial or total loss of specific bio-activity for the purified constituent. The natural constituent synergy becomes lost. Very often the chemical complexity of the crude or partially purified extract seems to be essential for the bioavailability of the active constituents. It has been observed that complexation with certain other clinically useful nutrients (like saponin, steroids *etc*.) substantially improves the bioavailability of such extracts and their individual constituents[26].

The present study showed that all the fractions more or less increased the life span than that of the EAC control. The reliable criteria for judging the value of any anticancer drug are prolongation of life span and decrease the WBC. Further more the reduced volume of EAC and increased survival time of mice suggest the delaying impact of all the fractions on cell division[27]. Usually in cancer chemotherapy the major problem is anemia due to reduction in RBC. Reduction in viable cell count and increased nonviable cell count towards normal in tumor host suggest antitumor effect against EAC cell in mice. These suggested that all the fractions have direct relationship with tumor cells as these tumor cells are absorbed the anticancer drug by direct absorption in peritoneal cavity and this anticancer agent lysis the cells by direct cytotoxic mechanism[28].

Fractions FlavSt-1 and FlavSt-2 showed significant ($p < 0.05$) decrease in tumor volume, viable cell count, tumor weight and elevated the life span of EAC tumor bearing mice. Hematological profile such as RBC, hemoglobin, WBC and lymphocyte count reverted to normal level in the treated mice. Antioxidant enzymes are considered to be the first line of cellular defense against oxidative damage. Among all antioxidant enzymes, LPO, CAT and GSH are most important antioxidant defense in nearly all cells exposed to oxygen[29]. In the present study, all the fractions restored the antioxidant



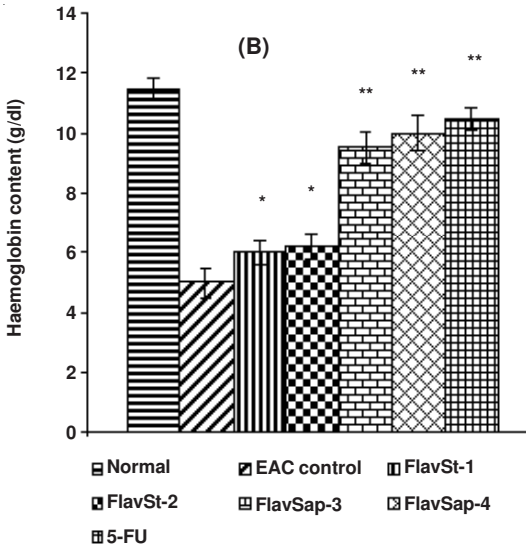




Fig. 2. Effect of the fractions hematological parameters of EAC treated mice. RBC count (cells × $10^6$ µL) (A), hemoglobin content (g/dl) (B) and WBC count (cells × $10^3$ µL) (C). Each point represents the mean ± SEM (n = 10 mice per groups) *$p < 0.05$ and **$p < 0.01$, 'a' indicates not significant

enzyme level towards more or less normal but the most significant one is FlavSap-3 and FlavSap-4. Biochemical parameter was also restored toward normal levels. But the significant value is increased ($p < 0.01$) due to presence of saponin along with flavonids and the activity is comparable with standard 5-fluorouracil. The cytotoxic effect is also significantly increased due to synergistic action of saponin with flavonoids.

## Conclusion

Finally it was concluded that the cytotoxicity and antitumor activity of the fractions were probably due to presence of some antioxidant flavonoids and its shows synergistic

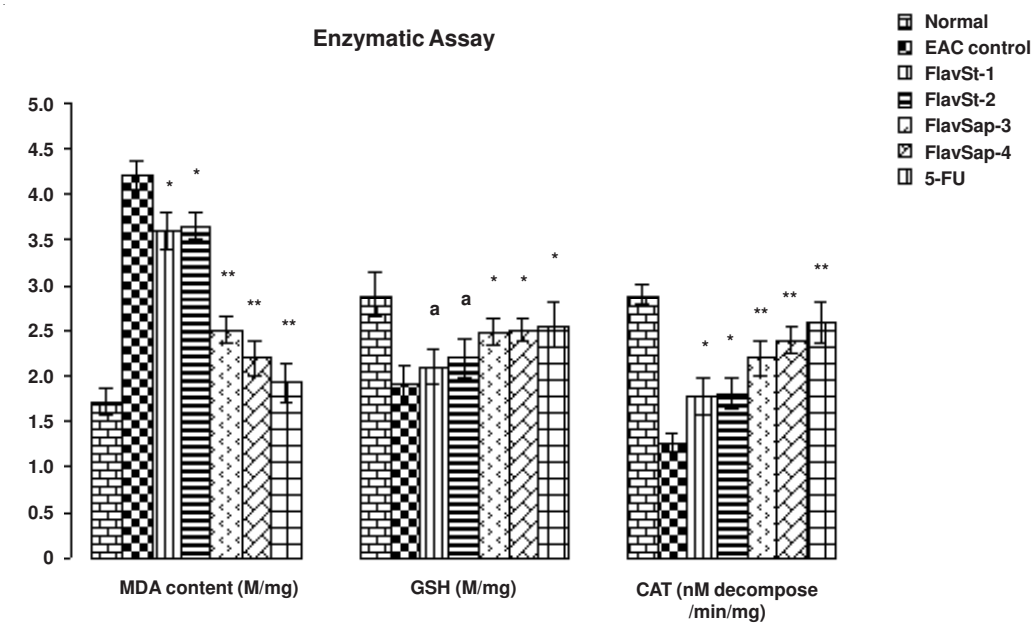



Fig. 3. Effect of all the fractions on enzymatic assay of EAC treated mice. Lipid peroxidation as well as MDA content, reduce glutathione (GSH) and Catalase (CAT) level. Each point represents the mean ± SEM (n = 10 mice per groups) *$p < 0.05$ and **$p < 0.01$, 'a' indicates not significant

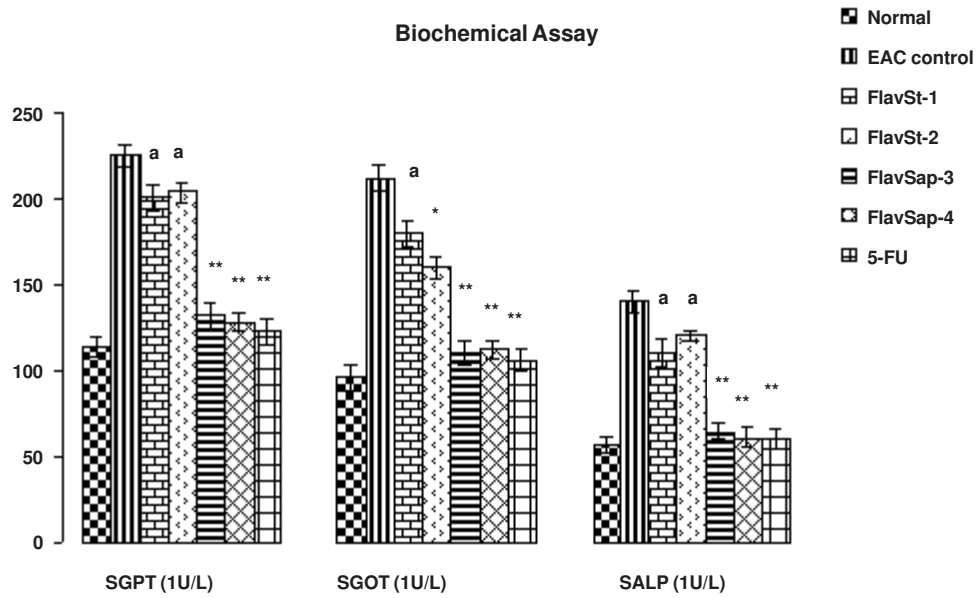



Fig. 4. Effect of all the fractions on biochemical assay of EAC treated mice. Serum glutamate-pyruvate transaminase (SGPT), serum glutamate-oxaloacetate transaminase (SGOT), serum alkaline phosphatase (SALP). Each point represents the mean ± SEM (n = 10 mice per groups) $*p < 0.05$ and $**p < 0.01$, 'a' indicates not significant

action along with saponin. Further purification and identification is going on in our laboratory for focusing a new compound in sight into the world of drug discovery and designing.

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